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TITLE: DISCOVERY AND DEVELOPMENT OF THERAPEUTIC DRUGS AGAINST LETHAL HUMAN RNA VIRUSES: A MULTIDISCIPLINARY ASSAULT

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13. ABSTRACT *(Maximum 200 words)*

Continuation of a vigorous investigation sharply focused on the isolation, characterization and structural elucidation of new and potentially useful antiviral drugs from marine animals and plants was pursued. The renewal approval allowed further progress toward the isolation and characterization of novel antiviral substances from confirmed active extracts of marine organisms and both higher and lower plants. The synthetic studies with narciclasine was continued and the supply of pancratistatin from both semi-synthetic and botanical sources was studied further.

14. SUBJECT TERMS

RNA Viruses; Antiviral Drug Discovery; Pancratistatin and related antiviral leads; RA I; BD; Natural Products

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FOREWORD

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[signature]	3/17/92
PI Signature	Date

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1. Background

This investigator's original proposal, submitted in 1987, reviewed the rapidly threatening medical consequences of the HIV family of retroviruses. Again, upon application for research support in 1990, the background summary continued the review from that period.

2. Hypothesis

Discovery and development of new antiviral drugs from extracts of terrestrial plants and marine organisms was the principal objective of our program in the government's medical research program.

3. Objectives

The ASU Cancer Research group has been completely dedicated to discovery and development of potentially useful anticancer drugs for 35 years and expanded that research 12 years ago to include discovery of drugs effective against RNA viruses. The financial support provided by the USAMRIID program was used to isolate and characterize new antiviral chemotherapeutic drugs from confirmed active extracts of marine invertebrates and vertebrates as well as marine and terrestrial plants including fungi, algae and other microorganisms. The continued research effort was sharply focused on marine animal and plant species yielding extracts with an outstanding level of antiviral activity in the USAMRIID's program (RNA viruses).

4. Military Significance

Lethal RNA viruses continue to pose a great and rapidly expanding threat to military personnel. The HIV-1 (AIDS) virus is now added to the known lethal encephalitises, hemorrhagic fevers and other viral diseases of unknown etiology.

5. Methodology

Experience gained in previous research involving discovery of new marine organism and terrestrial plant antineoplastic agents has been applied to the discovery of antiviral constituents from the same natural sources. Similar techniques utilizing extraction, partition and chromatographic separations have been applied and were followed with the new confirmed active antiviral leads.

6. Statement of Work

For the three-month interval June through September, the work scope was continued and focussed on further development of leads from confirmed active extracts from marine and plant species with very high and reproducible antiviral activity. The attached manuscript (in press) entitled "Antiviral (RNA) Activity of Selected Amaryllidaceae Isoquinoline Constituents and Synthesis of Related Substances" and the three attached reprints just published provide a major review of this past year's research accomplishments and progress. Termination of the Army's antiviral drug discovery program is a very serious matter and places our military personnel at greatly increased risk from these lethal RNA viruses. The research program was exceptionally successful and promising. Doubtlessly clinically useful antiviral drugs against the major RNA viruses would have been discovered and developed in this exceptionally valuable research endeavor. In addition to the splendid results noted especially in the attached manuscript concerning pancratistatin against Japanese Encephalitis, the following leads were pursued in the July → September 1991 report period:

Prescreen Submissions: (529 samples)

Date	Marines	Plants	Total
7/10/91	118	234	352
8/12/91	116	61	177

Samples Submitted to the Full Screen:

22 synthetics; 4 plant fractions and 2 marine fractions

7/5/91	GRP-19788	651.1 mg.	AVS-4277
	B848528 (FO15)	652.1 mg.	AVS-7087
	B619315 (FO12)	652.7 mg.	AVS-6986
8/19/91	B680433 (K488)	10.0 mg.	AVS-11940
9/23/91	B712568 (KO10)	8.9 mg.	AVS-11958
	GRP-18081	21.0 mg.	AVS-11959
	GRP-18082	23.6 mg.	AVS-11960
	GRP-18083	28.0 mg.	AVS-11961
	GRP-18084	10.0 mg.	AVS-11962
9/25/91	GRP-18085	10.1 mg.	AVS-11963
	GRP-18086	10.4 mg.	AVS-11964
	GRP-18087	10.6 mg.	AVS-11965
	GRP-18088	10.2 mg.	AVS-11966
10/14/91	GRP-23987	10.0 mg.	AVS-11968
	B849350 (KO18)	10.0 mg.	AVS-11967
10/17/91	GRP-18091 (GG-131)	11.4 mg.	AVS-11969
	GRP-18092 (GG-105)	10.0 mg.	AVS-11970
	GRP-18093 (G-133)	10.1 mg.	AVS-11971
	GRP-18094 (G-149)	10.0 mg.	AVS-11972
	GRP-18095 (G-181C)	1.9 mg.	AVS-11973
	GRP-18096 (G-189)	10.3 mg.	AVS-11974
	GRP-18097 (G-13C)	10.5 mg.	AVS-11975
	GRP-18098 (G-159A)	2.4 mg.	AVS-11976
	GRP-18099 (G-301-1)	10.0 mg.	AVS-11977
	GRP-18100 (G-301-2)	11.4 mg.	AVS-11978
	GRP-18101 (G-301-3)	10.3 mg.	AVS-11979
10/30/91	GRP-18103	10.3 mg.	AVS-11980
	B725014 (K110)	5.0 mg.	AVS-11981

7. Bibliography of Publications

Manuscript in press:

B. Gabrielsen, T. P. Monath, J. W. Huggins, D. F. Kefauver, G. R. Pettit, G. Groszek, M. Hollingshead, J. J. Kirsi, W. M. Shannon, E. M. Schubert, J. DaRe, B. Ugarkar, M. A. Ussery, and M. J. Phelan, "Antiviral (RNA) Activity of Selected Amaryllidaceae Isoquinoline Constituents and Synthesis of Related Substances," *J. Nat. Prod.*

Papers Published: (One copy of each attached)

G. R. Pettit, D. L. Doubek, D. L. Herald, A. Numata, C. Takahasi, R. Fujiki, and T. Miyamoto, "Isolation and Structure of Cytostatic Steroidal Saponins from the African Medicinal Plant *Balanites aegyptica*," *J. Nat. Prod.*, 54, 1491 (1991.

G. R. Pettit, C. L. Herald, M. R. Boyd, J. E. Leet, C. Dufresne, D. L. Doubek, J. M. Schmidt, R. L. Cerny, J. N. A. Hooper, and K. C. Rützler, "Isolation and Structure of the Cell Growth Inhibitory Constituents from the Western Pacific Marine Sponge *Axinella* sp.," *J. Med. Chem.*, 34, 3339 (1991).

G. R. Pettit, D. Sengupta, C. L. Herald, N. A. Sharkey, and P. M. Blumberg, "Synthetic Conversion of Bryostatin 2 to Bryostatin 1 and Related Bryopyrans," *Can. J. Chem.*, 69, 856 (1991).

8. Personnel Receiving Pay

Name	% FTE	Period
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Dr. Grazyna Groszek	100	7/1/91 - 9/30/91
Dr. Rui Tan	100	7/1/91 - 9/30/91
Dr. Bruce E. Tucker	100	7/1/91 - 9/30/91

J. Nat. Prod.
Accepted

ANTIVIRAL (RNA) ACTIVITY OF SELECTED AMARYLLIDACEAE ISOQUINOLINE CONSTITUENTS AND SYNTHESIS OF RELATED SUBSTANCES

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£ Deceased, 1991.

ABSTRACT

A series of 23 Amaryllidaceae isoquinoline alkaloids and related synthetic analogues were isolated or synthesized and subsequently evaluated in cell culture against the RNA-containing flaviviruses (Japanese encephalitis, yellow fever, and dengue viruses), bunyaviruses (Punta Toro, sandfly fever, and Rift Valley fever, viruses), alphavirus (Venezuelan equine encephalomyelitis virus), lentivirus (human immunodeficiency virus-type 1) and the DNA-containing vaccinia virus. Narciclasine **1**, lycoricidine **2**, pancratistatin **4**, 7-deoxypancratistatin **5** and acetates **6-8**, isonarciclasine **13a**, cis- and trans-dihydronarciclasines **14a**, **15a**, 7-deoxy analogues **13b-15b**, lycorines **16**, **17**, and pretazettine **18** exhibited consistent in vitro activity against all three flaviviruses and against the bunyaviruses, Punta Toro and Rift Valley fever virus. Activity against sandfly fever virus was only observed with 7-deoxy analogues. In most cases however, selectivity of the active compounds was low, with toxicity in uninfected cells (TC_{50}) occurring at concentrations within 10-fold that of the viral inhibitory concentrations (IC_{50}). No activity was observed against human immunodeficiency virus type-1, Venezuelan equine encephalomyelitis virus or vaccinia viruses. Pancratistatin **4** and its 7-deoxy analogue **5** were evaluated in two murine Japanese encephalitis mouse models (differing in viral dose

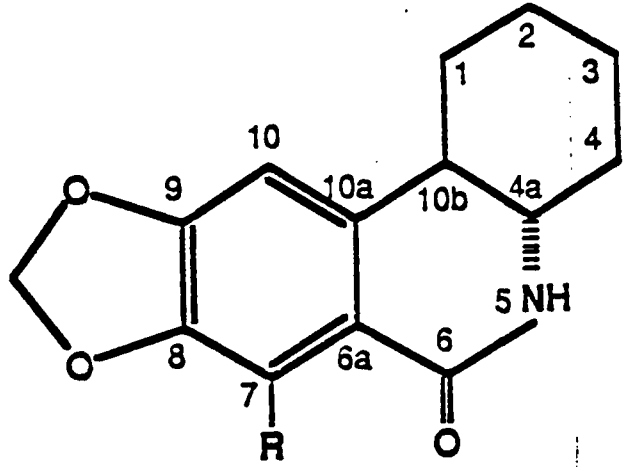
challenge among other factors). In two experiments (low LD_{50} viral challenge, variant I), prophylactic administration of **4** at 4 and 6 mg/kg/day (2% ethanol-saline, s.c., QID X 7, day -1 to +5) increased survival of Japanese encephalitis virus-infected mice to 100% and 90%, respectively. In the same model, prophylactic administration of **5** at 40 mg/kg/day in hydroxypropylcellulose (s.c., QID X 7, day -1 to +5) increased survival of Japanese encephalitis virus-infected mice to 80%. In a second variant (high LD_{50} viral challenge), administration of **4** at 6 mg/kg/day (i.p., BID X 9, day -1 to +7) resulted in a 50% survival rate. In all cases, there was no survival in the diluent-treated control mice. Thus, **4** and **5** demonstrated activity in mice infected with Japanese encephalitis virus but only at near toxic concentrations. To our knowledge, however, this represents a rare demonstration of chemotherapeutic efficacy (by a substance other than an interferon inducer) in a Japanese-encephalitis virus-infected mouse model.

[1] This paper was presented in part at the 4th Chemical Congress of North America, New York, N.Y., August 25-30, 1991.

INTRODUCTION

Numerous chemotherapeutic agents have their origins in terrestrial plant and marine organism sources (1-3). For example, plants of the family Amaryllidaceae have yielded over 100 different alkaloids with diverse biological properties (2). The medicinal properties of extracts from the daffodil *Narcissus poeticus* L. (Amaryllidaceae) were known to the Greek physician Hippocrates of Cos as early as 300 B.C. and to Romans of the first century AD. Over the past two decades, a number of potentially important constituents of this family have been identified as isocarbostyrils such as narciclasine, **1** [whose extraction and antimitotic properties have been described by Ceriotti (4)]; lycoricidine (7-deoxynarciclasine) **2**; pancratistatin (5-7) **4**; and the alkaloids lycorine **16**, pseudolycorine **17**, and pretazettine, **18**. Pancratistatin **4** and 7-deoxynarciclasine **2** have been isolated from the bulbs of Hawaiian *Pancratium littorale*. Jacq. and *Zephyranthes grandiflora* and their structures elucidated (7). Considerable efforts (8,9) have recently culminated in the total synthesis of racemic pancratistatin by Danishefsky and Lee (8). Trans-dihydronarciclasine, **15a**, the principal antiviral and cytostatic constituent of the Chinese medicinal plant, *Zephyranthes candida*, has also been recently isolated (10). The numbered ring structure for compounds including **1**, **2**, and **4** is depicted in Figure 1.

Figure 1. Numbered narciclasine / pancratistatin ring structure.


2
1
3
4
10
9
10a
10b
4a
5 NH
6
8
6a
7
R
O
O

Biological properties, syntheses and mechanisms of action (if known) of the narciclasine- and lycorine-type Amaryllidaceae alkaloids have been summarized (2,3,11-15). For example, lycorine **16** was found to be responsible for the antiviral activity of leaf and root extracts of the Amaryllidaceae plant, *Clivia miniata Regel* against Herpes simplex, Semliki forest, polio, Coxsackie, and measles viruses in Vero cells (16). Such biosynthetic products exert their biological activities mainly by inhibiting protein synthesis at the step of peptide bond formation. A recent study of the effect of lycorine on viral protein formation in poliovirus-infected HeLa cells serves as a useful illustration (17). Pseudolycorine **17** and pretazettine **18** similarly inhibit protein synthesis and have manifested activity against murine Rauscher leukemia virus and neurotropic RNA viruses (18). While some reports (19) describe the antiviral properties of crude extracts containing the isocarbostyril-type compounds against neurotropic RNA viral infections in mice with Japanese encephalitis virus and lymphocytic choriomeningitis (LCM) virus (20-22), to our knowledge, no systematic study has been undertaken to evaluate the antiviral (RNA) activities of the pure, naturally occurring compounds and synthetic derivatives. Structure-activity studies have been limited by the synthetic strategies required.

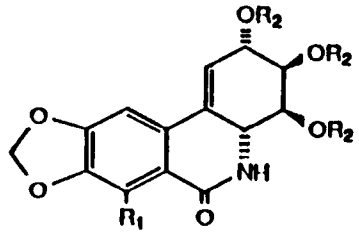
A group of RNA viruses belonging to the families Flaviviridae, Bunyaviridae, and Togaviridae cause hemorrhagic, encephalitic, or febrile disease throughout large areas of the world (23). Vaccines exist to control some flavivirus diseases (24-26);

however, with the exception of interferon and its inducer poly(ICLC)[1] (27), no specific antiviral chemotherapeutic agents have demonstrated efficacy against flaviviral infections (28). Ribavirin, 1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide, already approved for therapy against respiratory syncytial virus in children, has shown efficacy against some bunyavirus infections (29,30). Our preliminary evaluation of certain Amaryllis constituents gave antiviral screening data that indicated the presence of *in vitro* activities against Flaviviridae and Bunyaviridae. In order to explore this lead, compounds **1-18** (see Figure 2) have been obtained and their antiviral (RNA) activities evaluated both *in vitro* and in appropriate available animal models.

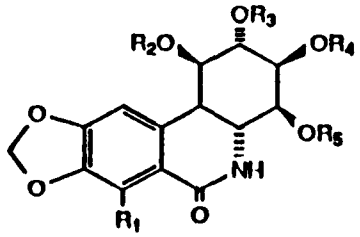
[1] a nuclease-resistant complex of polyriboinosinic-polyribocytidylic acid, poly-l-lysine and carboxymethylcellulose.

Place here Figure 2. Compounds Synthesized / Isolated for Antiviral Evaluation.

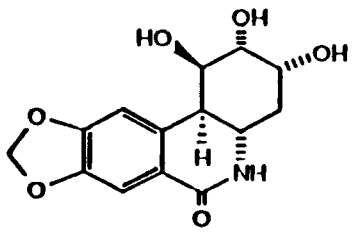
Compounds Synthesized or Isolated for Antiviral Evaluation



1: Narciclasine; R_1=OH; R_2=H
2: 7-Deoxynarciclasine (lycoricidine); R_1=R_2=H
3: Lycoricidine triacetate; R_1=H; R_2=Ac



4: Pancratistatin; R_1=OH; R_2 – R_5=H
5: R_1 – R_5=H
6: R_1=R_2=R_4=R_5=H; R_3=Ac
7: R_1=H; R_2 – R_5=Ac
8: R_1=R_2=H; R_3=R_4=R_5=Ac



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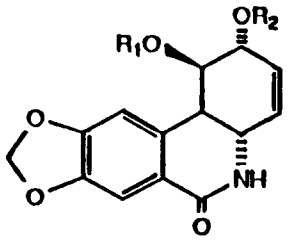
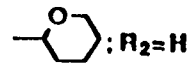
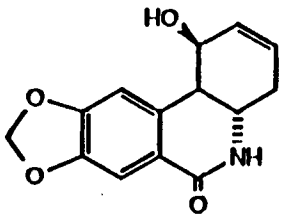


10: R_1=  ; R_2=H

11: R_1=R_2=H



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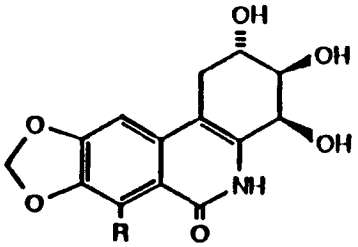
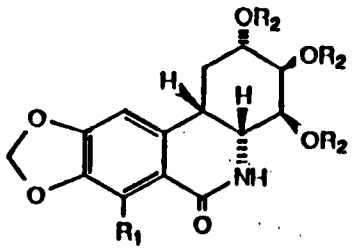
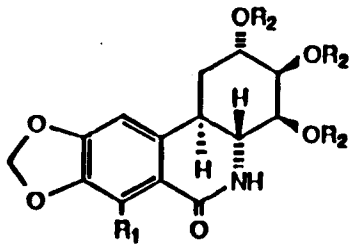


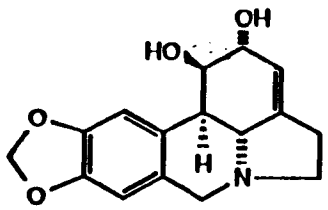
13a: Isonarciclasine; R=OH
b: Iso-7-deoxynarciclasine; R=H



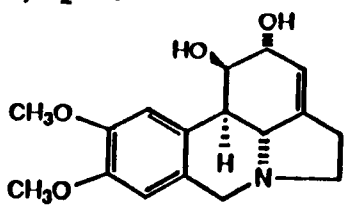
14a: cis-Dihydronarciclasine; R_1=OH; R_2=H
b: R_1=R_2=H
c: R_1=OAc; R_2=Ac
d: R_1=H; R_2=Ac



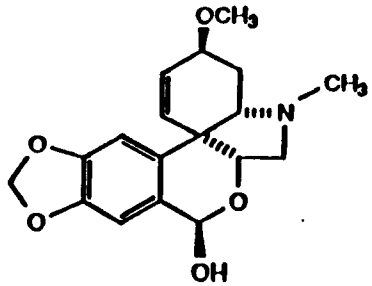
15a: trans-Dihydronarciclasine; R_1=OH; R_2=H
b: R_1=R_2=H
c: R_1=OAc; R_2=Ac
d: R_1=H; R_2=Ac



16: Lycorine



17: Pseudolycorine



18: Pretazettine

RESULTS AND DISCUSSION

Chemistry

Compounds **1-18** used in this study were either synthesized, isolated from natural sources, or furnished for testing as acknowledged (see Experimental).

Narciclasine **1** and lycorine **16** are obtained by extraction of the bulbs of *Narcissus incomparabilis* (31). Syntheses have been reported for (+)-lycoricidine (32,33), for racemic lycorine (34-38), and pancratistatin (8) as well as for the isonarciclasines **13a,b** (39-41). An elegant, highly-convergent synthesis of racemic lycorine has been reported (42) based in part upon synthetic strategy described by Stork (43). For this study, 7-deoxynarciclasine **2** (lycoricidine) and pancratistatin **4** were isolated from *Pancratium littorale* (7). No specific synthesis of narciclasine has appeared but techniques used for isolating pancratistatin (8) were very effective. Lycoricidine triacetate **3**, monoacetate **6**, triacetate **8**, and hydroxy analogues **10-12** were synthesized by methods initially described by Ohta and Kimoto (44) and subsequently modified by Schubert (45). 7-Deoxypancratistatin **5** (44,46) and triol **9** were obtained by synthesis from intermediates **10** and **12**. Acetylation of **5** with acetic anhydride and 4-dimethylaminopyridine (DMAP) gave tetraacetate **7** (44).

Isonarciclasine (**13a**) and the <u>cis</u>- and <u>trans</u>-dihydronarciclasines (**14a, 15a**) were obtained from narciclasine by catalytic hydrogenation on Adam's catalyst, as described by Mondon and Krohn (47) with modifications described herein. The

double bond-isomerized, sparingly-soluble product, isonarciclasine, **13a**, was isolated when pyridine was used in place of dimethylformamide as solvent. The mixture of cis- and trans-dihydronarciclasines, **(14a, 15a,)**, present in the pyridine filtrate, were acetylated with acetic anhydride to afford a mixture of the tetraacetates, **14c, 15c**. Purification by column chromatography on silica gel and removal of the acetate protecting groups (31) by heating with 2N aqueous barium hydroxide solution gave the pure cis- and trans-dihydronarciclasines. The trans-dihydronarciclasine was also isolated from *Zephyranthes candida* (10). When 7-deoxynarciclasine **(2)** was treated by a similar hydrogenation/acetylation procedure, 7-deoxy-isonarciclasine **(13b)** and the 7-deoxy-cis- and trans-dihydronarciclasines **(14b, 15b)** were obtained from triacetates, **14d, 15d**. The enantiospecific total synthesis of trans-dihydro-lycoricidine **15b** was recently reported (48). The spectroscopic properties of the synthetic analogues have been established by 300 MHz and 2-dimensional (COSY) NMR spectroscopy (see Experimental).

Antiviral Activity

***In-vitro* studies.** The isolated alkaloids narciclasine **1**, 7-deoxynarciclasine **2**, pancratistatin **4**, lycorines **16-17**, and pretazettine **18** and synthetized analogues **3, 5-15**, were evaluated to determine their in vitro inhibitory properties against the RNA-containing flaviviruses [Japanese encephalitis, (JE), yellow fever, (YF), and dengue type 4 viruses]; bunyaviruses [Punta Toro, (PT), sandfly fever-Sicilian, (SF), and Rift Valley fever (RVF)

viruses]; the alphavirus (family Togaviridae), Venezuelan equine encephalomyelitis (VEE) virus, the lentivirus, human immunodeficiency virus type 1 (HIV-1), and the DNA-containing vaccinia virus (VV). The antiviral assays used determine the 50% inhibition (IC_{50}) of virus-induced cytopathic effect by an MTT assay (53,54) except for dengue and RVF viruses, where activity was determined by a plaque reduction assay (50,51,55). The concentration of test compound which was cytotoxic to 50% of uninfected cells (TC_{50}) was also determined, as was the ratio of these two values (TI), expressed as a therapeutic index.

In general, antiviral activity was consistently observed against the flaviviruses tested (JE, YF, dengue-4) and to a slightly lesser degree against the bunyaviruses (PT, SF, RVF). The IC_{50}, TC_{50}, and TI data are summarized in Tables I and II. All compounds tested had anti-flavivirus activity except lycoricidine triacetate **3** (which could be considered a prodrug of **2**), triol **9**, alcohols **10-12**, and 7-deoxy-isonarciclasine **13b** (vs. JE virus). Generally, however, the selectivity of the agents was low. Toxicity in uninfected cells (TC_{50}) generally occurred at concentrations within 10-fold (or less) that of the viral inhibitory concentration (IC_{50}). Exceptions included the mono- and triacetates **6** and **8**, which could be considered precursors of 7-deoxypancratistatin **5**. No activity was observed against VEE, HIV-1 or VV (data not shown).

The pattern of *in-vitro* activity exhibited against all flaviviruses was not observed against the three bunyaviruses. Most compounds **1-18** inhibited PT and RVF viruses but generally with low selectivity. Pancratistatin **4** failed to reduce the viral cytopathic effect by 50% against PT virus. Acetate precursors **3** and **7**, triol **9**, and alcohols **10-12** were inactive against RVF and PT viruses. Less activity was observed against SF virus. Only 7-deoxynarciclasine **2**, 7-deoxypancratistatin **5**, 7-deoxy-*cis*-dihydronarciclasine **14b**, and pretazettine **18** showed marginal activity ($TI_{50}<4.5$). It is of interest to note that of the four compounds exhibiting activity against SF virus, all lack the C-7 hydroxy group.

Further structure/activity correlations are also evident. Whereas narciclasine **1** and its 7-deoxy analogue **2** were the most toxic to host cells of any compounds in this series (in addition to pancratistatin **4** and the *trans*-dihydro analogues **15a,b**), shifting of the double bond from the C_1-C_{10b} position of the C-ring to the C_{10b}-C_{4a} position (isonarciclasines **13a,b**) increased the TC_{50} values by 24-200 fold. This change is reflected in the fact that isonarciclasine **13a** exhibited the highest selectivity (TI_{50}) against dengue virus of any of the unacetylated compounds. Similarly, the presence of a *cis*-fused C-ring (**14a-c**) in place of a *trans*-fused C-ring (**15a-c**) resulted in decreases in toxicity (increased TC_{50}) by factors of 200-280. The presence or absence of a 7-hydroxy substituent was also manifested in the TC_{50} values.

When comparing pancratistatin **4** with its 7-deoxy analogue **5** (and similarly narciclasine **1** with lycoricidine **2**), host cell toxicity (TC_{50}) was reduced by 8-32-fold when the 7-hydroxy substituent was replaced by hydrogen. Successive esterification of the hydroxy groups at C_1-C_4 (compounds **5-8**) also served to increase the TC_{50} values with increasing esterification. Finally, the effects of C-ring hydroxylation on antiviral efficacy were ascertained by comparing triol **9**, diol **11**, and mono-hydroxy **12**, with 7-deoxypancratistatin **5**. Only the latter compound **5** showed any antiviral activity. Thus, if one disregards the configurational change of the C_3 hydroxy group in **5** and **9**, then the presence of a hydroxy group at C_4 becomes necessary for antiviral activity.

***In-vivo* studies.** Pancratistatin **4** and its 7-deoxy analog **5** were evaluated in one or both of the variants of the murine JE virus models described (see Experimental). In variant I (low viral dose challenge model, $9LD_{50}$), prophylactic administration of 7-deoxypancratistatin **5** in hydroxypropylcellulose (HPC) at 40 mg/kg/day (s.c. QID x 7, day -1 to +5) significantly ($p=3.6 \times 10^{-4}$) increased survival to 80% in virus-infected mice compared to the diluent-treated control animals (0% survival). At 20 mg/kg/day, an increase in survival rate to 60% and a prolonged average day of death (ADD) were observed while a dose of 10 mg/kg/day was ineffective in reducing mortality or increasing the ADD. Toxicity control animals receiving 40 mg/kg/day lost weight during the treatment period; no toxicity was observed at lower doses (see

Table III). Thus, prophylaxis with 7-deoxypancratistatin partially protected against JE viral infections in mice. However, efficacy was primarily observed at dose levels approaching toxicity. Pancratistatin 4 exhibited similar results. Prophylactic administration of a 2%-ethanolic-saline solution of pancratistatin with variant I (see Table III) at 4 mg/kg/day (2% ethanol-saline, s.c., Q1D x 7, day -1 to +5) resulted in 100% survival of JE virus-infected mice as compared to the diluent-treated controls (0% survival). At 6 mg/kg/day, pancratistatin was toxic to 60% of the uninfected mice while a dose of 2 mg/kg/day did not significantly reduce JE virus-induced mortality. However, it did prolong the average day of death (ADD). In a second experiment using the same model, 4 was administered at 6 mg/kg/day, and significantly increased survival (to 90%) in JE virus-challenged mice compared to the diluent treated controls (0% survival). No mortality occurred in the toxicity control animals. At 4 mg/kg/day, survival was reduced to 30% while the 2 mg/kg/day dose level had no significant effect on the JE viral challenge.

In variant II (high viral challenge model, 100 LD_{50}), prophylactic administration of pancratistatin (see Table IV) at 2-8 mg/kg/day, (i.p., BID, 9 days, day -1 to +7) resulted in only 50% survival at the 6 mg/kg/day dose as compared to 0% survival in the diluent-treated mice. While this represents a significant increase in the number of survivors, there was no significant increase in the mean time to death of the non-surviving animals,

although the value corresponding to the 8 mg/kg dose approached significance.

Insolubility of pancratistatin in aqueous media and limited bioavailability may be partially responsible for poor reproducibility of pharmacokinetics which may have produced the variable in vivo test results. Differences between the two murine models include age of mice, administration routes of drug and virus, as well as schedule, and the LD_{50} of viral challenge. We did not determine which of these differences accounted for the experimental variations. However, these results are significant in that they represent one of the few examples of chemotherapy of JE viral infections in animal systems. [One earlier study (20) describes the effects of narcissidine, 4-methoxy-pseudolycorine (22), derived from *Narcissus tazetta*, on JE virus-infected mice, which had prolonged survival times but there were no significant differences in mortality of treated versus control animals.] Further syntheses and evaluations of additional analogues are underway in an effort to broaden the therapeutic margin between efficacy and toxicity.

Conclusions. We found that a series of naturally-occurring or synthetic Amaryllidaceae alkaloids (1-18) related to narciclasine and lycorine possess antiviral efficacy with accompanying low selectivity in vitro against three flaviviruses, JE, YF and dengue viruses; against the bunyaviruses, PT and RVF viruses; and, to a lesser extent, against SF virus.We found prophylactic efficacy of

pancratistatin and 7-deoxypancratistatin in JE virus-infected mice; however, chemotherapeutic doses bordered on toxic doses. Therefore, further structure/activity-guided synthetic studies will be pursued in an effort to produce a more effective compound with less toxicity.

EXPERIMENTAL

All solvents were distilled before use, dried when necessary, and all chemicals were reagent grade. Evaporations were conducted at bath temperatures ≤30° with a Buchi rotary evaporator under water aspirator or mechanical oil pump vacuum. Melting points were determined with a Thomas Hoover capillary apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA. Results agreed within ±0.4% of theory. The ^{1}H nmr and ^{13}C nmr spectra were recorded on a Varian XL200 spectrometer with ADVANCE data system operating at 200 MHz and 50.3 MHz, respectively. Additional ^{1}H nmr spectra were recorded on a Nicolet NT300WB spectrometer with 1280 data system operating at 300 MHz. Standard Varian COSY determined proton-proton connectivity. Mass spectral data were obtained by employing a MAT 312 mass spectrometer. Chemical shifts are expressed in parts per million and referenced to tetramethylsilane (^{1}H nmr) or dimethylsulfoxide at 39.7 ppm (^{13}C nmr). IR spectra were recorded by a Perkin Elmer Model 1310 spectrophotometer as Nujol mulls or KBr pellets. Thin layer chromatography was performed on Woelm F silica gel sheets (254/366) with detection of products under a short wavelength UV lamp and/or spraying with 40% methanolic H_2SO_4 and charring.

Pseudolycorine **17** and pretazettine **18** were obtained from both the National Cancer Institute and the University of Hawaii. Narciclasine **1**, lycoricidine **2**, pancratistatin **4**, and lycorine **16** were obtained by isolation (7,31). Lycoricidine analogues **3**, **6**, **8**, **10**, **11** and **12** were prepared by Schubert's modification (45) of the earlier procedure of Ohta and Kimoto (44). Isonarciclasine **13a**, cis- and trans-dihydronarciclasines **14a**, **15a**, and their respective tetraacetates **14c**, **15c** were synthesized from narciclasine according to procedures described by Mondon and Krohn (39-41,47).

4aH-r,1H-trans,2H-cis,3H-trans,4H-trans,10bH-trans,1,2,3,4-Tetrahydroxy-8,9-methylenedioxy-1,2,3,4,4a,10b-hexahydro-6(5H)-phenanthridone (7-deoxypancratistatin, 5). Osmium tetroxide (100 mg, 0.39 mmol) was added to a solution of N-methylmorpholine-N-oxide (2.2 g, 19 mmol) dissolved in t-butanol (25 mL), acetone (25 mL) and water (20 mL), and the mixture stirred for 10 min. A warm solution of racemic intermediate (45) **10** (4 g, 11 mmol) in t-butanol/acetone (200 mL each) was added over 5 min. The mixture was stirred for 24 h at 25° at which point TLC (6:1 $CHCl_3$/MeOH) indicated ca.60% reaction. Additional osmium tetroxide (50 mg, 0.195 mmol) and N-methyl-morpholine-N-oxide (500 mg) were added. After 24 h (95% completion), the solvents were removed in vacuo at 35°. Trituration of the residue with ethanol (25 mL), removal of solvent in vacuo, followed by a second treatment with ethanol (25

mL) and filtration gave the 1-(2'-tetrahydropyranyloxy)-2,3,4-trihydroxy analogue as a pale-yellow solid, 3.6 g (82%), mp 222-224°. Without further purification, the tetrahydropyranyl group was removed by heating at reflux a mixture of the pyranyl ether (3.4 g, 8.6 mmol) and p-toluenesulfonic acid monohydrate (200 mg) in ethanol (150 mL) for 3 h. Cooling to 0°, filtration, concentration of the filtrate in vacuo, followed by a second cooling and filtration, gave two crops of tetraol 5. Recrystallizations from acetic acid followed by water gave 1.51 g (57%) of alcohol 5 as an analytically-pure, off-white amorphous powder, mp 308° (dec.), [lit.(44) mp 305°]. NMR/IR spectral data agreed with literature values (44).

4aH-r,1H-trans,2H-cis,3H-trans,4H-trans,10bH-trans,1,2,3,4-Tetraacetoxy-8,9-methylenedioxy-1,2,3,4,4a,10b-hexahydro-6(5H)-phenanthridone (7-deoxypancratistatin-1,2,3,4-tetraacetate, 7). A solution of the 1-(2'-tetrahydropyranyloxy)-2,3,4-trihydroxy analogue prepared above (3.4 g, 9.5 mmol) and p-toluenesulfonic acid monohydrate (500 mg) in ethanol (300 mL) was heated at reflux for 12 h. The volume was reduced in vacuo to 40 mL and the mixture was cooled. The resulting product was collected by filtration, dissolved in acetic anhydride (200 mL) containing 4-dimethylaminopyridine (DMAP, 400 mg) and the solution heated at reflux for 10 h. Acetic anhydride was removed by distillation under reduced pressure followed by co-evaporation with ethanol (2 x 50 mL). The solid residue was recrystallized from acetone (200

mL) yielding 2.1 g (51%) of acetate 7, mp 306° [lit.(44) mp 300°). NMR/IR spectral data agreed with literature values (44).

4aH-r,1H-trans,2H-cis,3H-cis,10bH-trans,1,2,3-Trihydroxy-8,9-methylene-dioxy-1,2,3,4,4a,10b-hexahydro-6(5H)phenanthridone (9). Osmium tetroxide (12.5 mg, *0.048 mmol) was added to a solution of* N-methylmorpholine-N-oxide (0.78 g, 6.6 mmol) in t-butanol (2.4 mL), acetone (2.4 mL), and water (1 mL). A solution of olefin (44) **12** (1 g, 3.86 mmol) in 50% t-butanol/acetone was added over 5 min. The reaction mixture was stirred at 25° for 6 days until TLC (6:1, $CHCl_3$/MeOH) showed complete disappearance of starting material **12**. The solution was treated with charcoal, stirred for 3 h at 25°, filtered through Celite (prepared in aqueous acetone) and solvent removed in vacuo. Two recrystallizations from water gave 620 mg (49%) of triol **9**, mp 288-290°; ^1H nmr (DMSO-d_6); d 7.69 (s, 1, exchanges with D_2O, NH), 7.32 (s, 1, H-7, uncoupled in COSY), 6.91 (s, 1, H-10, coupled to H-10b), 6.06 (AB, J = 0.96 Hz, 2, CH_2O), 5.12, 4.87, 4.67 (d, J = 4.7, 3.3, 6.4 Hz, respectively, 1 each, exchange with D_2O, C_1-OH, C_2-OH, C_3-OH, respectively), 4.37 (d, J = 2.5 Hz, 1, H-1, coupled to H-2, H-10b, C_1-OH), 3.86 (dd, 1, H-3, coupled to H-2, H-4), 3.81 (dd, overlapping partially with H-3, 1, H-2, coupled to H-1, H-3), 3.55 (dt, J = 12.4, 3.8 Hz, 1, H-4a, coupled to H-4, H-10b), 2.85 (dd, J = 12.6, 1.5 Hz, 1, H-10b, coupled to H-10, H-4a, H-1), 1.8 (dd, $J_{ea} = J_{ae} = 3.8$ Hz, 1, H-4 equatorial), 1.7 (dd, $J_{aa} = 11.7$ Hz, 1, H-4 axial); ^{13}C nmr (tentative assignments): d 164.04 (C=O), 150.15, 145.63, 135.60, and 124.23 (aryl);

106.75 and 105.21 (C-7,C-10); 101.34 (CH_2O), 72.13, 68.03, and 65.30 (C-1,C-2,C-3); 47.85 (C-4b); 40.15 (C-10b); and 33.89 (C-4). Anal. Found: %C, 57.25, H, 5.19, N, 4.74; $C_{14}H_{15}NO_6$ requires: %C, 57.34, H, 5.16, N, 4.78;

7-Deoxy-isonarciclasine (13b), 7-deoxy-cis and trans-dihydro-narciclasine triacetates (39-41) (14d, 15d). A solution of 7-deoxynarciclasine **2** (1.02 g, 3.4 mmol) in a 1:1 mixture of methanol and ethanol (400 mL) was degassed with nitrogen followed by addition of platinum oxide (57 mg). The mixture was hydrogenated at ambient temperature and pressure for 24 h producing a precipitate which was collected with catalyst by filtration through Celite. The residue was heated in pyridine and the solution filtered through Celite. Concentration of the filtrate in vacuo gave a dark brown solid (150 mg), which crystallized from pyridine-hexane to give 7-deoxy-isonarciclasine (39-41) **13b**, m.p.>300° (100 mg, 9.8%); IR (KBr): 3400, 3284, 3237, 1653, 1629, 1590, 1487, 1472, 1460, 1064, 1042 cm^{-1}; ^1H nmr (DMSO-d_6): d 1.95, 1.99, 2.12 (brs, 1H each, OH), 2.38 (dd, J = 16.2, 6.5 Hz, 1, H-1), 2.94 (dd, J = 16.2, 5.3, 1, H-1), 3.61 (dd, J = 7.6, 3.3 Hz, 1, H-3), 4.00 (dd, J = 12.9, 6.3 Hz, 1, H-2), 4.50 (d, J = 2.2 Hz, 1, H-4), 6.16 (brs, 2, OCH_2O), 7.1 (s, 1, H-7), 7.54 (s, 1, H-10), and 8.58 (brs, 1, NH). ^{13}C nmr (DMSO-d_6): d 30.33 (C-1), 66.02 (CH), 66.21 (CH), 71.74 (CH), 101.19 (C-7), 101.84 (CH_2), 104.41 (C-10), 106.11 (C-10b), 120.71 (C-4a), 134.60 (C-6a, C-10a), 146.7 (C-8), 151.68 (C-9), and 160.70 (C-

6). EIMS (m/z) : 291 (M^+, 100%), 275 (20), 255 (32), 244 (20), 231 (28), 203 (55).

The original filtrate obtained after removing precipitate and catalyst was concentrated to dryness *in vacuo* and treated with acetic anhydride (7 mL) - pyridine (10 mL) at 60° for 6 h. Pyridine was removed by azeotropic distillation with methanol and cyclohexane and the residue was concentrated to dryness. Flash chromatography on a silica gel column and elution with methylene chloride - methanol (99.4:0.6) followed by crystallization from acetone-hexane gave 150 mg (10%) of 7-deoxy-*trans*-dihydronarciclasine triacetate (40) **15d**, mp 148-9°. IR (KBr): 3420, 1757, 1661, 1502, 1474, 1462, 1245, 1224, 1190, 1061, 1038 cm^{-1}; ^1H nmr ($CDCl_3$): d 1.92 (m, 1, H-1), 2.08 (s, 3, $COCH_3$), 2.13 (s, 3, CH_3), 2.14 (s, 3, CH_3), 2.48 (m, 1, H-1), 3.17 (dt, J = 12.6, 4.2 Hz, 1, H-10b), 3.82 (dd, J = 12.6, 10.7 Hz, 1, H-4a), 5.18 - 5.45 (m, 3, $CHOCOCH_3$), 6.03 (s, 2, OCH_2O), 6.72 (s, 1, H-10), 6.94 (brs, 1, NH), 7.52 (s, 1, H-7). EIMS (m/z): 419 (M^+, 40%), 360 (30), 297 (20), 255 (98), 239 (100). Continued elution of the column yielded a mixture of *cis* and *trans* triacetates **14d** and **15d** (374 mg, 26%) followed by pure 7-deoxy-*cis*-dihydronarciclasine triacetate (40) **14d**, 440 mg (31%). Crystallization from acetone-hexane gave an amorphous powder, mp 144-6°. IR (NaCl): 3300, 1750, 1671, 1481, 1467, 1371, 1246, 1231, 1053, 1038, 756 cm^{-1}; ^1H nmr ($CDCl_3$): d 1.80 - 2.30 (m, 1, H-1), 2.01 (s, 3, $COCH_3$), 2.04 (s, 3, $COCH_3$), 2.16 (s, 3, $COCH_3$), 3.20 (dt, J = 14.7, 3.8 Hz, 1, H-

10b), 3.92 (t, J = 3.8 Hz, 1, H-4a), 5.20 - 5.56 (m, 3, CHOCOCH$_3$), 6.02 (s, 2, OCH$_2$O), 6.24 (brs, 1, NH), 6.67 (s, 1, H-10), 7.84 (s, 1, H-7). EIMS (m/z): 419 (M$^+$, 15%), 299 (20), 257 (42), 239 (100).

7-Deoxy-trans-dihydronarciclasine (15b). A mixture of 7-deoxy-trans-dihydronarciclasine triacetate **15d** (60 mg, 0.14 mmol) in methanol (15 mL) and aqueous 1M barium hydroxide solution (5 mL) was heated on a steam bath for 15 min, cooled to 25°, saturated with solid carbon dioxide, stirred at 25° overnight, and filtered. The filtrate was evaporated to dryness and the residue redissolved in methanol and the solution filtered. The solvent was removed *in vacuo* and the residue crystallized from methanol to give 35 mg (83.4%) of 7-deoxy-trans-dihydronarciclasine **15b**, mp 320-2° [lit.(40) mp >300°]; IR (KBr): 3555, 3491, 3454, 3427, 1671, 1464, 1268, 1074, 1047, 1036 cm^{-1}; ^{1}H nmr (DMSO-d$_6$): d 1.63 (dt, J = 13.0, 2.4 Hz, 1, H-1), 2.13 (dt, J = 13.0, 3.0 Hz, 1, H-1), 2.872 (dt, J = 12.0, 3.6 Hz, 1, H-10b), 3.30 (m, 1, H-4a, signal flanked with water in DMSO), 3.705 (brs, 2, 2 x OH), 3.869 (brs, 1, OH), 4.825 (brd, J = 3.3 Hz, 1, -CHOH), 4.940 (brd, J = 5.8 Hz, 1, -CHOH), 4.976 (brd, J = 3.4 Hz, 1, -CHOH), 6.065 (brs, 2, OCH$_2$O), 6.924 (s, 1, H-10), 6.930 (brs, 1, NH), 7.290 (s, 1, H-7). EIMS (m/z): 293 (M$^+$, 72%), 202 (100), 189 (60).

7-Deoxy-cis-dihydronarciclasine (14b). A solution of 7-deoxy-cis-dihydronarciclasine triacetate **14d** (440 mg, 1.05 mmol) in methanol (50 mL) containing potassium carbonate (200 mg) was stirred at 25° for 2 h,

then filtered through a Sephadex LH-20 column. The column was eluted with methylene chloride-methanol (3:2), and the product was crystallized from methanol-acetone to give 7-deoxy-cis-dihydronarciclasine **14b** (250 mg, 81%) as an amorphous powder, mp >300° [lit.(40) mp >300°]; IR (KBr): 3300, 1653, 1610, 1468, 1404, 1387, 1357 cm^{-1}; ^1H nmr (DMSO-d_6): d 1.300 (q, J = 12.5 Hz, 1, H-1), 1.685 (dt, J = 12.5, 4.0 Hz, 1, H-1), 3.030 (dt, J = 12.5, 4.0 Hz, 1, H-10b), 3.300 (m, 1, H-4a, signal flanked with water in DMSO), 3.595 (t, J = 3.6 Hz, 1, -CHOH), 3.638 (dd, J = 11.0, 4.5 Hz, 1, -CHOH), 3.929 (t, J = 3.0 Hz, 1, -CHOH), 4.400-5.100 (brs, 3, 3 x OH), 6.040 (m, 2, OCH_2O), 6.949 (s, 1, H-10), 7.231 (s, 1, H-7), 7.751 (brs, 1, NH). EIMS (m/z): 293 (M^+, 50%), 202 (45), 189 (18).

***In-Vitro* Antiviral and Cytotoxicity Assays.** The *in-vitro* antiviral and cytotoxic effects of a test compound were measured (49) either: a) by observing inhibition of viral cytopathic effect (50-52) by using an MTT-assay [JE, YF, SF, PT, VEE, VV, and HIV-1 viruses (53,54)] or b) by a general plaque reduction assay [dengue virus (55), RVF virus (56)]. Compounds were evaluated for antiviral efficacy against the following viruses (viral strain): a) JE virus, (Nakayama); B) YF virus, (Ashibi); c) SF virus, (Sicilian); d) PT virus, (Adames); e) VEE virus, (Trinidad donkey); f) VV ,(Lederle vaccine). All assays were carried out in Vero cells except for the use of MT-2 and CEM cells in the HIV-1 assay (53,54). Compounds to be assayed were dissolved in dimethylsulfoxide (DMSO) and diluted with water to a final volume of 2%

except for **1**, **2**, **5-8**, **14c**, **15c**, which were solubilized in aqueous ethanol.

A general plaque reduction assay (55,56) was used to test for antiviral activity of candidate compounds against dengue virus. Each drug to be tested was dissolved in appropriate diluent (DMSO, ethanol), brought to twice the highest concentration to be tested in cell culture maintainance medium (Hank's basal salt solution-Hepes containing 2% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 10 ug/mL streptomycin), and sterilized by filtration. Five two-fold dilutions of 2x drug were prepared in cell culture medium. For each drug, 12 wells of a 24-well plastic tissue culture plate containing confluent monolayer cultures of LLCMK2 cells were used. Six wells were infected by removing growth medium and adding 100 µL of dengue 4 virus (Caribbean strain) containing 50-100 plaque-forming units (pfu). The remaining six wells received medium without virus. After adsorption for 1 h, 0.5 mL each 2x concentration of drug was added to duplicate (infected and control) wells. Medium without drug was added as a control. Cultures were then overlaid with 2.5% agarose in nutrient medium and incubated for 6 days, at which time they were stained by adding of 2 mL of 5% neutral red. Wells were decanted after 4 h and plaques counted. The IC_{50} was determined as the concentration of drug reducing plaques by 50% over the untreated control, while the minimum toxic concentration (MTC) was estimated visually by inspection of uninfected drug-treated wells.

Basic measurements and definitions used throughout these studies include: (a) Cellular toxic concentration 50% (TC_{50}), is defined as the drug concentration (Ug/mL) that reduces cell numbers and their metabolic activity by 50% as compared to the viability of uninfected control cells in duplicate test wells in the MTT assay. (b) Viral inhibitory concentration 50% (IC_{50}) is defined as the drug concentration (ug/mL) at which 50% reduction of viral cytopathic effect (CPE) is observed in triplicate test wells. The therapeutic (or antiviral) index (TI), is a value proportional to the overall in-vitro activity and is calculated as a ratio of (TC_{50}/IC_{50}). It is a single drug concentration measurement of the relative anticellular and antiviral effectiveness of a compound during the same test and time period. All in-vitro MTT assay results given represent an average of two-six individual test results.

***In-vivo* studies. Murine Japanese Encephalitis Virus Model, Variant I. Low LD_{50} Viral Challenge.** Groups of 15 C57Bl/6 mice (VAF+, Charles River Labs.) weighing 14 to 16 g, were treated s.c. with phosphate-buffered saline (PBS) or drug once daily on a 7-day schedule with the first dose administered on the day (day -1) preceding viral challenge. Ten of the 15 animals in each group were infected i.p. with 9 LD_{50} (adequate to produce 90-100% mortality in the diluent controls) of JE virus (Beijing strain) 2 h after the second dose of compound was administered (day 0). Controls included untreated, uninfected mice; untreated, virus-infected mice; diluent-treated, virus-infected mice; and diluent-treated, uninfected mice. Pancratistatin was solubilized in

ethanol and diluted in sterile saline to a final ethanol concentration of 2%; 7-deoxypancratistatin was suspended in NCI-hydroxypropylcellulose, HPC. Compounds were prepared at concentrations appropriate for dosing at 0.1 ml of compound per 10 g of body weight. Animals were monitored for 28 days post-virus infection. Body weights were recorded on days -1 through 7, 14 and 21. Weight change was determined as a measure of drug toxicity. The average day of death (ADD) and geometric mean time to death (GMTD) were calculated. An in-vivo virus rating (VR) for each drug concentration was calculated by dividing the geometric mean time to death (GMTD) in treated animals by that in diluent-treated controls. The statistical significance of differences in the mortality rates for the drug-treated, virus-infected animals compared to the diluent-treated, virus-infected animals was compared by Fishers' Exact test. Differences in the ADD for drug-treated, virus-infected animals compared to the diluent-treated, virus-infected animals were compared by Student's t-test.

Variant II. High LD_{50} Viral Challenge. Groups of 15 C57B1/6 mice (VAF+, Charles River Labs.) weighing 12-14 g were treated i.p. with phosphate-buffered saline (PBS) or drug twice daily (b.i.d.) on a 9-day schedule with the first dose administered on the day (day -1) preceding viral challenge. Ten of the 15 animals in each group were infected s.c. with 100 LD_{50} of JE virus (Beijing strain, adequate to produce 100% mortality in the diluent controls) 6 h after the second dose of compound was administered (day 0). Controls included untreated, uninfected mice; untreated, virus-infected mice; diluent-treated virus-infected (and

uninfected) mice. Animals were monitored for 28 days post-virus infection. Body weights were recorded on days -1 through +7. Weight change was determined as a measure of drug toxicity.

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Yellow Fever and Dengue - Type 4

Virus :	Japanese Encephalitis[a]			Yellow Fever[a]			Dengue - 4[b]		
Compound	TC_{50}[c]	IC_{50}[c]	TI_{50}	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}
1	0.031	0.008	4.08	0.037	0.006	6.1	0.06	0.015	4.0
2	0.27	0.056	4.9	0.29	0.053	5.6	0.25	0.059	4.2
3	>1.0	Inactive		>1.0	Inactive		>25	Inactive	
4	0.092	0.022	4.2	0.079	0.016	4.9	0.5	0.063	8.0
5	2.8	0.48	5.9	2.6	0.4	6.6	2.5	0.67	3.7
6	10.8	3.3	3.3	21.0	4.8	4.3	50.0	1.5	33.3
7	2020	724	2.8	>1000	262	3.8	not tested		
8	16.4	4.5	3.6	8.3	2.2	3.8	100	<5.0	>20.0
9	2000	Inactive		1800	Inactive		not tested		
10	>100	Inactive		>100	Inactive		not tested		
11	630	Inactive		500	Inactive		not tested		
12	>100	Inactive		>100	Inactive		not tested		
13a	1.5	0.72	2.1	0.90	0.22	4.1	5.0	0.27	18.5
13b	>100	Inactive		23.8	5.7	4.2	50.0	8.5	5.9
14a	4.9	0.96	5.1	5.2	1.3	3.9	>5.0	2.5	>2.0
14b	62.5	12.7	4.9	64.0	9.6	6.6	25.0	4.4	5.7
14c	29.0	8.1	3.5	97.0	d	d	not tested		
15a	0.025	0.004	5.6	0.027	<0.003	>8.5	0.063	0.015	4.2
15b	0.22	0.039	5.6	0.28	0.037	7.5	2.5	0.5	5.0
16	2.7	0.33	8.2	2.04	0.28	7.3	2.5	0.24	10.4
17	1.4	0.28	5.0	1.3	0.35	3.7	1.0	0.39	2.6
18	2.3	0.60	3.8	2.8	0.50	5.6			

[a] TC_{50} and IC_{50} obtained by MTT assay.

[b] IC_{50} measured by plaque reduction; TC_{50} measured by cytopathic effect.

[c] In ug / mL.

[d] Viral cytopathic effect reduced 25-49% only.

Table II. Antiviral Activity In Vitro Against Bunyaviruses - Punta Toro, Rift Valley Fever and Sandfly Fever - Sicilian

Virus :	Punta Toro [a]			Rift Valley Fever [b]			Sandfly Fever - Sicilian [a]		
mpound	TC_{50} [a]	IC_{50} [a]	TI_{50}	TC_{50}	IC_{50}	TI_{50}	TC_{50}	IC_{50}	TI_{50}
1	0.029	0.0074	3.9	0.022	Inactive		0.028	Inactive	
2	0.27	0.042	6.3	0.83	0.15	5.5	0.26	0.058	4.5
3	>1.0	Inactive		<2.5	Inactive		>1.0	Inactive	
4	0.10	d	d	0.5	0.16	3.1	0.13	Inactive	
5	2.9	0.66	4.3	21.5	5.1	4.3	4.5	1.7	2.7
6	9.6	4.7	2.1	24.0	5.5	4.4	5.2	Inactive	
7	>320	Inactive		>250	Inactive		>320	Inactive	
8	10.6	2.5	4.2	<250	Inactive		21.5	Inactive	
9	1200	Inactive		>250	Inactive		>320	Inactive	
10	>100	Inactive		<250	Inactive		>100	Inactive	
11	500	Inactive		<250	Inactive		>320	Inactive	
12	>320	Inactive		250	Inactive		>320	Inactive	
13a	1.4	0.28	5.1	25.0	3.3	7.6	0.72	Inactive	
13b	26.2	7.2	3.7	50.0	10.0	5.0	17.6	Inactive	
14a	8.0	2.2	3.6	5.0	1.4	3.6	8.0	d	d
14b	68.0	14.0	4.8	not tested			73.0	25	3.0
14c	77.0	12.0	6.4	not tested			21	d	d
15a	0.026	0.008	3.3	not tested			0.027	Inactive	
15b	0.34	0.057	5.9	0.5	0.25	2.0	0.25	Inactive	
16	2.3	0.50	4.6	5.0	0.93	5.4	1.4	Inactive	
17	2.3	0.60	3.9	3.8	0.63	6.0	2.5	Inactive	
18	2.3	0.61	3.7	10.0	2.9	3.5	2.5	0.82	3.0

[a] TC_{50} and IC_{50} obtained by MTT assay.

[b] IC_{50} measured by plaque reduction; TC_{50} measured by cytopathic effect.

[c] In ug / mL.

[d] Viral cytopathic effect reduced 25-49% only.

Table III. Evaluation of Pancratistatin 4 and 7-Deoxypancratistatin 5 in the Murine Japanese Encephalitis (Low Virus Dose Challenge) Model

Treatment	Dose (mg / kg / day)	Uninfected (#Dead / Total)	[a] ADD ± SD	Infected (#Dead / Total)	[b] p	[a] ADD ± SD	[c] p	[d] GMTD
pancratistatin	6	3 / 5	2.7 ± 0.6	7 / 10	0.105	4.0 ± 2.4	[e] ND	6.5
	4	0 / 5		0 / 10	<0.001	—	NA	28.0
	2	0 / 5		8 / 10	0.24	16.3 ± 1.9	<0.001	18.0
untreated		0 / 5		10 / 10	[f] NA	14.0 ± 1.8	NA	13.9
2% ethanol-saline		0 / 5		10 / 10	NA	12.8 ± 1.3	NA	12.7
pancratistatin	6	0 / 5		1 / 10	<0.001	15.0 ± 0.0	0.36	26.3
	4	0 / 5		7 / 10	0.105	15.4 ± 2.0	0.02	18.4
	2	1 / 5	28.0 ± 0.0	9 / 10	0.50	11.6 ± 1.4	ND	12.5
untreated		0 / 5		10 / 10	NA	12.2 ± 2.5	NA	11.9
2% ethanol-saline		0 / 5		10 / 10	NA	12.4 ± 2.6	NA	12.1
7-deoxypancratistatin	40	0 / 5		2 / 10	<0.001	9.5 ± 4.9	0.06	22.2
	20	0 / 5		4 / 10	0.005	15.0 ± 2.6	0.03	21.7
	10	0 / 5		9 / 10	0.50	13.6 ± 1.9	0.21	14.4
Untreated		0 / 5		10 / 10	NA	12.2 ± 2.5	NA	11.9
Hydroxypropylcellulose		0 / 5		10 / 10	NA	12.6 ± 1.2	NA	12.6

[a] Average day of death ± standard deviation
[b] p value by Fisher's Exact Test comparing mortality in drug-treated to diluent-treated mice
[c] p value by Student's t-test comparing days of death in drug-treated to diluent-treated mice
[d] Geometric mean time to death
[e] ND - not done
[f] NA - not applicable

Table IV. Evaluation of Pancratistatin 4 in the Murine Japanese Encephalitis (High Virus Dose Challenge) Model

Treatment	Dose (mg / kg / day)	Wt. Change (Day +7)	Uninfected (#dead / total)	Infected (# dead / total)	ADD ± SD (days)
Pancratistatin	8	1.5	2 / 5	7 / 10	12.9 ± 0.5
	6	1.3	1 / 5	5 / 10 *	11.8 ± 0.1
	5	1.4	1 / 5	7 / 10	10.4 ± 0.5
	4	1.8	0 / 5	9 / 9	12.3
	3	2.1	0 / 5	9 / 10	12.6
	2	2.8	0 / 5	9 / 10	12.6
Untreated		2.7	0 / 10	10 / 10	11.5
2% ethanol-saline		1.8	0 / 10	10 / 10	12.6

* Significant at p=0.05; 2-tailed compared to 2% ethanol-saline treatment. Survival by Fisher's Exact Test; average day of death (ADD) by t-test.

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ISOLATION AND STRUCTURE OF CYTOSTATIC STEROIDAL SAPONINS FROM THE AFRICAN MEDICINAL PLANT *BALANITES AEGYPTICA*[1]

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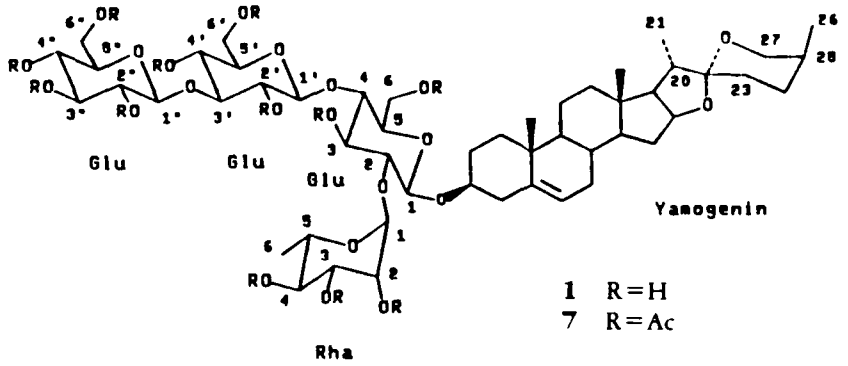
ABSTRACT.—Bioactivity-guided separation of a CH_2Cl_2/MeOH extract of *Balanites aegyptica* afforded four new cytostatic saponins, named balanitins 4 [**1**], 5 [**2**], 6 [**3**], and 7 [**4**]. On the basis of enzymatic hydrolyses and glycosidation nmr chemical shifts employing the peracetates, structures **1**–**4** were established as yamogenin 3β-*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [**1**], yamogenin 3β-*O*-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [**2**], yamogenin 3β-*O*-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [**3**], and diosgenin 3β-*O*-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside [**4**].

Balanites aegyptica Del. (Balanitaceae) is widely employed in Africa as a component of various primitive medicines used for abortifacient, antiseptic, antiviral (herpes zoster), antimalarial, molluscicidal, antisyphilitic, and vermifuge purposes (2,3). In 1982 the molluscicidal steroidal saponins balanitins 1–3, isolated from the root and bark of this plant, were reported (4). As part of our evaluation of higher plant antineoplastic and/or cytostatic constituents, we examined the seed components of this plant using the murine P-388 lymphocytic leukemia (PS system) in cell culture (5). A 1978 re-collection (107 kg) of seeds was crushed and extracted using CH_2Cl_2/MeOH (followed by dilution with H_2O) (6,7). The aqueous phase was successively partitioned (8) between MeOH-H_2O (9:1→4:1→1:1)→H_2O and hexane→CCl_4→CH_2Cl_2→*n*-BuOH. The most encouraging PS activity (ED_{50} 17 μg/ml) was located in the *n*-BuOH fraction. Bioassay-directed fractionation employing (8) Sephadex LH-20, Si gel, cc, and hplc afforded four new PS cytostatic constituents **1**–**4** designated balanitins 4–7 (4).

The mol wt of balanitin 6 [**3**] was deduced as 884 from the peaks at *m/z* 885 $[M + H]^+$ and 907 $[M + Na]^+$ in the fabms. In the ir spectrum, glycoside **3** exhibited absorption bands at 988, 920, and 900 cm^{-1} with the 920 band stronger than the 900 band, characteristic of a 25(*S*)-spirostan (9, 10) along with hydroxyl group absorptions at 3430 and 3280 cm^{-1}. Comparison of the ^1H- and ^{13}C-nmr data of glycoside **3** with published values (4, 11) suggested a glycoside of yamogenin (see Experimental and Table 1). A choice between yamogenin and diosgenin as the aglycone was based on the chemical shifts of H-27, C-23, and C-27 in the ^1H- and ^{13}C-nmr spectra, respectively, plus the ir bands near 988–900 cm^{-1}. Acetylation of glycoside **3** afforded nonaacetate **5**. The ^1H-nmr signals in the sugar moiety of this derivative were assigned on the basis of the ^1H-^1H homonuclear decoupling technique. That established the presence of two glucose and one rhamnose units (Table 2).

The anomeric configurations of glucose and xylose in the balanitins were deduced as β from the large J ^1H-^1H coupling and small J ^{13}C-^1H coupling values (12) of each anomeric position displayed by the peracetate derivatives. The rhamnose orientation

[1]Number 196 in the series Antineoplastic Agents. For Part 195 see Singh and Pettit (1).



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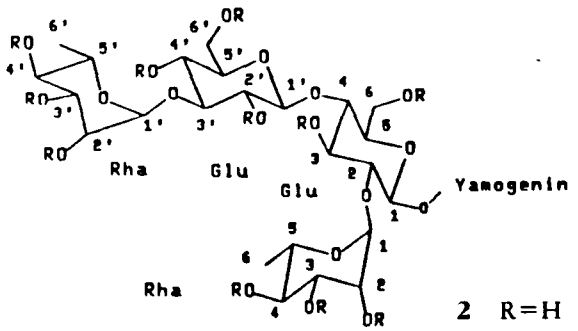
7 R=Ac



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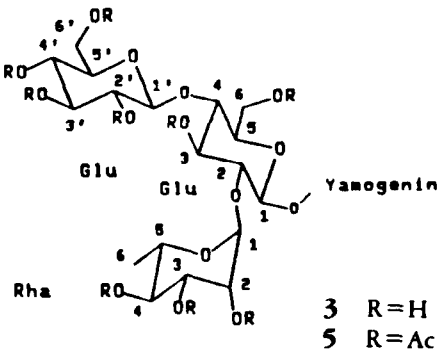

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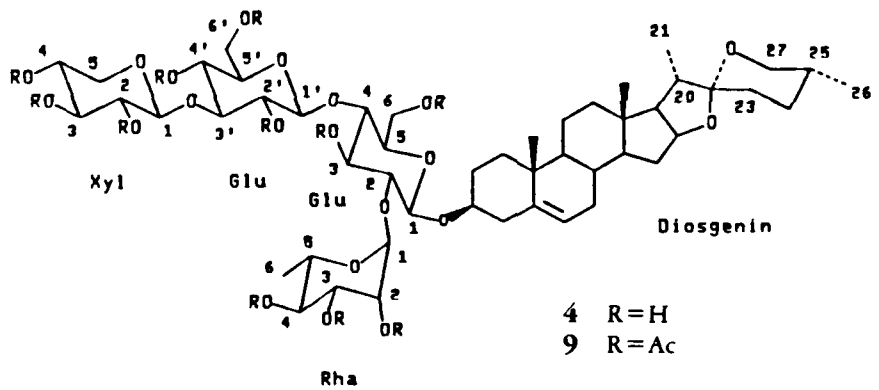

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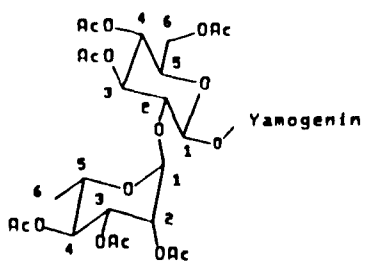
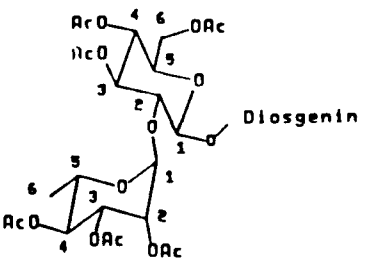
9 R=Ac



6



10

was determined as α from the large J ^{13}C-^{1}H coupling value for the anomeric carbon in the corresponding peracetates. Also, good agreement was found between the ^{13}C-nmr chemical shifts (especially for C-3 and C-5 which are easily distinguishable from a β configuration) (13) in those peracetates with the values for methyl *O*-triacetyl-α-L-rhamnopyranoside (Table 3).

Once the glycoside configurations were established, the sequence was next under-

TABLE 1. ^{13}C-nmr Chemical Shifts (δ ppm) of Balanitins 4 [**1**], 5 [**2**], 6 [**3**] and 7 [**4**] in pyridine-d_5.

Carbon	Compound			
	1	**2**	**3**	**4**
C-1	37.48	37.52	37.50	37.51
C-2	29.98	30.12	30.14	30.15
C-3	78.31	78.29	78.29	78.27
C-4	38.92	38.92	38.92	38.95
C-5	140.78	140.81	140.80	140.81
C-6	121.78	121.78	121.78	121.81
C-7	32.32	32.20	32.32	32.24
C-8	31.70	31.70	31.70	31.71
C-9	50.31	50.29	50.30	50.31
C-10	37.16	37.16	37.13	37.15
C-11	21.11	21.10	21.10	21.13
C-12	39.84	39.87	39.86	39.87
C-13	40.43	40.47	40.45	40.48
C-14	56.62	56.65	56.64	56.65
C-15	32.20	32.31	32.21	32.24
C-16	81.18	81.18	81.18	81.12
C-17	62.71	62.75	62.74	62.93
C-18	16.34	16.37	16.32	16.35
C-19	19.38	19.43	19.40	19.42
C-20	42.46	42.46	42.46	42.00
C-21	14.90	14.90	14.89	15.06
C-22	109.73	109.73	109.72	109.27
C-23	26.38	26.42	26.40	31.86
C-24	26.18	26.22	26.21	29.30
C-25	27.54	27.53	27.54	30.63
C-26	65.08	65.07	65.08	66.88
C-27	16.34	16.37	16.32	17.34
G-1	101.75	101.78	101.79	101.80
G-6	61.74[a]	61.79[a]	62.09[a]	61.74[a]
G-1′	104.59	104.75	105.23	104.61
G-6′	61.47[a]	61.56[a]	61.96[a]	61.52[a]
G-1″	105.93			
G-6″	62.53			
R-1	99.95	99.98	100.00	99.98
R-6	18.66	18.63	18.66	18.68
R-1′		102.87		
R-6′		18.63		
X-1				106.36
X-5				62.75
Other sugar units	69.32	69.21	69.45	67.44
	69.46	69.47	71.21	69.01
	71.60	69.88	72.44	69.49
	72.47	72.41	72.77	70.95
	72.78	72.50	74.13	72.47
	73.70	72.64	74.95	72.79
	74.09	72.74	76.19	74.01
	75.56	74.10	77.28	74.16
	76.27	75.14	77.73	75.35
	77.21	76.22	78.12	76.29
	77.65	77.26	78.48	77.25
	78.01	77.65	82.07	77.66
	78.12	78.15		78.14
	78.71	81.33		81.51
	81.48	83.04		87.33
	88.33			

[a]Assignments may be interchanged.

TABLE 2. ^{1}H-nmr Chemical Shifts and Coupling Constants of Balanitins 6 Peracetate [**5**], 4 Peracetate [**7**], 5 Peracetate [**8**], and 7 Peracetate [**9**] in Pyridine-d_5.

Proton	Compound							
	5		**7**		**8**		**9**	
	δ ppm	J ^{1}H-^{1}H	δ ppm	J ^{1}H-^{1}H	δ ppm	J ^{1}H-^{1}H	δ ppm	J ^{1}H-^{1}H
H-3	3.92 m		3.93 m		3.95 m		3.93 m	
H-6	5.52 d	5.0	5.56 d	5.0	5.54		5.55 d	5
H-16	4.51 q	7.0	4.53[a]		4.57 q	7.0	4.55[a]	
H-18	0.87 s		0.88 s		0.88 s		0.89 s	
H-19	1.09 s		1.10 s		1.10 s		1.10 s	
H-21	1.17 d	7.0	1.17 d	6.9	1.17 d	6.9	1.16 d	6.8
H-27	1.09 d	6.5	1.09 d	6.9	1.09 d	6.7	0.71 d	5.3
G-1	4.88 (+0.05)[b] d	8.2	4.79 (−0.04) d	8	4.87 (+0.01) d	8	4.80 (−0.03) d	8
2	*4.03 (−1.43)*[b] dd	8.2, 10	*4.00 (−1.46)* dd	8, 10	*4.03 (−1.43)* dd	8, 10	*4.01 (−1.45)* dd	8.8, 9.9
3	5.74 (+0.01) t	10	5.68 (−0.05) t	10	5.74 (+0.01) t	10	5.68 (−0.05) t	9.9
4	*4.15 (−1.35)* t	10	*4.09 (−1.41)* t	10	*4.13 (−1.37)* t	10	*4.31 (−1.19)* t	9.9
5	3.91 (−0.19) m		3.85 (−0.25) m		3.93 (−0.17) m		3.86 (−0.24) m	
6	4.52 (+0.12) dd	6.4, 12	4.53 (+0.13) dd	6, 11	4.57 (+0.17) dd	6, 12	4.55 (+0.15) dd	5.2, 11
	4.84 (+0.27) br d	12	4.81 (+0.24) br d	11	4.84 (+0.27) br d	12	4.81 (+0.24) br d	11.6
G-1′	5.09 (+0.26) d	8.8	4.87 (+0.04) d	7.8	4.92 (+0.09) d	8.1	4.86 (+0.03) d	8.4
2′	5.42 (−0.04) dd	8.8, 10	5.43 (−0.12) dd	7.8, 10	5.45 (−0.01) dd	8.1, 9.8	5.40 (−0.06) dd	8.4, 10
3′	5.71 (−0.02) t	10	*4.48 (−1.25)* t	10	*4.24 (−1.49)* t	9.8	*4.31 (−1.42)* t	10
4′	5.50 (0) t	10	5.37 (−0.13) t	10	5.40 (−0.10) t	9.8	5.36 (−0.14) t	10
5′	4.16 (+0.06) m		4.04 (−0.06) m		3.99 (−0.11) m		4.02 (−0.08) m	
6′	4.33 (−0.13) dd	3, 12.4	4.32 (−0.08) dd	2, 12.4	4.32 (−0.08) br d	14	4.28 (−0.12) br d	12
	4.84 (+0.35) dd	5.2, 12.4	4.69 (+0.12) dd	4.2, 12.4	4.65 (+0.08) dd	5, 14	4.65 (+0.08) dd	4, 12
G-1″			5.15 (+0.32) d	8.2				
2″			5.38 (−0.08) dd	8.2, 9.9				
3″			5.75 (+0.02) t	9.9				
4″			5.46 (−0.04) t	9.9				
5″			4.07 (−0.03) m					
6″			4.20 (−0.20) dd	2.2, 12.4				
			4.65 (+0.08) dd	4.2, 12.4				

R-1	5.51 d	2	5.49 d	2.2	5.51 d	2	5.49 d	1.5
2	5.63 dd	2, 4	5.61 dd	2.2, 3.6	5.63 dd	2, 3.4	5.62 dd	1.5, 4
3	5.84 dd	4, 10	5.84 dd	3.6, 10.5	5.85 dd	3.4, 11	5.84 dd	4, 9.9
4	5.68 t	10	5.68 t	10.5	5.69 t	11	5.68 t	9.9
5	4.90 m		4.88 m		4.89 m		4.88 m	
6	1.50 d	6.3	1.45 d	6.3	1.51 d	6.3	1.50 d	6.3
R-1′					5.22 d	1.7		
2′					5.51 dd	1.7, 3.2		
3′					5.56 dd	3.2, 11.1		
4′					5.54 t	11.1		
5′					4.13 dq	6.3, 11.1		
6′					1.35 d	6.3		
X-1							5.01 d	6.9
2							5.29 dd	6.9, 8.7
3							5.64 t	8.7
4							5.22 dt	6, 8.7
5							4.29 dd	6, 12
6							3.63 dd	8.7, 12
CH_3CO	2.00 (6H)		2.00		1.96		1.98	
	2.02		2.017		2.01		2.017	
	2.04		2.02 (6H)		2.02		2.022	
	2.05 (6H)		2.036 (6H)		2.04		2.03	
	2.18		2.043		2.05		2.04	
	2.21		2.181 (6H)		2.08		2.05	
	2.42		2.184		2.10		2.08	
			2.32		2.12		2.16	
			2.41		2.18		2.18	
					2.45		2.32	
					2.48		2.40	

[a]Overlapping signals.
[b]Values in parentheses denote downfield (+) or upfield (−) shifts as compared to methyl tetraacetylglucoside.
[c]Assignments may be interchanged.
[d]Values in italics represent atoms involved in the glycoside linkages.

taken. The fabms of glycoside **3** gave ions at *m/z* 761 $[M + Na - 146]^+$ and 739 $[M + H - 146]^+$ due to loss of one rhamnose unit, and fragments at *m/z* 745 $[M + Na - 162]^+$ and 723 $[M + H - 162]^+$ arising from the loss of one glucose unit (Table 4) (4). Hence, both rhamnose and glucose were found linked to glucose in turn attached to yamogenin. The points of attachment of the sugar residues were based on glycosidation shifts (14,15) observed in the peracetate **5**. The ^{13}C-nmr signals in the

TABLE 3. ^{13}C-nmr Chemical Shifts (δ ppm) of Balanitins 6 Peracetate [**5**], 4 Peracetate [**7**], 5 Peracetate [**8**], and 7 Peracetate [**9**] in Pyridine.

Carbon	Compound			
	5	**7**	**8**	**9**
C-1	37.42	37.44	37.42	37.45
C-2	29.97	29.96	29.95	29.93
C-3	78.61	78.51	78.54	78.48
C-4	38.63	38.59	38.59	38.56
C-5	140.39	140.39	140.37	140.36
C-6	122.42	122.49	122.46	122.45
C-7	32.19	32.32	32.31	32.20
C-8	31.67	31.68	31.67	31.65
C-9	50.30	50.33	50.30	50.30
C-10	37.14	37.17	37.15	37.15
C-11	21.18	21.21	21.19	21.19
C-12	39.90	39.93	39.90	39.90
C-13	40.48	40.50	40.48	40.49
C-14	56.66	56.68	56.66	56.66
C-15	32.30	32.21	32.21	32.29
C-16	81.18	81.18	81.18	81.09
C-17	62.74	62.78	62.75	62.92
C-18	16.41	16.42	16.41	16.40
C-19	19.36	19.39	19.36	19.36
C-20	42.48	42.49	42.48	41.99
C-21	14.89	14.92	14.89	15.04
C-22	109.76	109.78	109.73	109.28
C-23	26.39	26.41	26.40	31.81
C-24	26.22	26.23	26.21	29.28
C-25	27.56	27.57	27.56	30.61
C-26	16.32	16.33	16.31	17.34
C-27	65.40	65.13	65.10	66.88
G-1	99.22(−2.67)[a][163.0][b]	99.10(−2.79)[162.4]	99.13(−2.76)[162.0]	99.08(−2.81)[162.1]
2	76.54(+4.55)[c]	76.48(+4.49)	76.53(+4.54)	76.46(+4.47)
3	74.80(+1.22)	74.70(+1.12)	74.80(+1.22)	74.68(+1.10)
4	77.58(+8.43)	77.24(+8.09)	77.33(+8.18)	77.19(+8.04)
5	73.31(+1.09)	73.35(+1.13)	73.38(+1.16)	73.29(+1.07)
6	62.74[d](+0.35)	62.85[d](+0.46)	62.75[d](+0.36)	62.80[d](+0.41)
G-1′	101.47(−0.42)[162.0]	101.44(−0.45)[162.2]	101.44(−0.45)[162.5]	101.44(−0.45)[162.1]
2′	72.30(+0.31)	73.48(+1.49)	72.64(+0.65)	73.29(+1.30)
3′	73.65(+0.07)	79.77(+6.19)	81.80(+8.22)	80.48(+6.90)
4′	68.56(−0.59)	68.21(−0.94)	69.70(+0.55)	68.42(−0.73)
5′	72.44(+0.22)	72.01[d](−0.21)	72.42(+0.20)	72.42(+0.20)
6′	62.08[d](−0.31)	62.09[d](−0.30)	62.23[d](−0.16)	62.27[d](−0.12)
G-1″		101.52(−0.37)[162.0]		
2″		71.80(−0.19)		
3″		73.67(+0.09)		
4″		68.72(−0.43)		
5″		72.46[d](+0.24)		
6″		62.21[d](−0.18)		
R-1	97.87(−0.91)[174.5]	97.82(−0.96)[176.1]	97.83(−1.02)[174.0]	97.79(−1.06)[176.0]
2	71.01(+1.02)	71.04(+1.05)	71.01(+0.84)	71.01(+0.84)
3	69.40(−0.27)	69.40(−0.27)	69.38(−0.48)	69.41(−0.45)
4	71.64(+0.58)	71.65(+0.59)	71.64(+0.41)	71.62(+0.39)
5	67.11(+0.56)	67.13(+0.58)	67.11(+0.40)	67.10(+0.39)
6	17.69(+0.33)	17.70(+0.34)	17.69(+0.04)	17.67(+0.02)
R-1′			99.95(+1.10)[172.3]	
2′			71.01(+0.84)	
3′			70.29(+0.43)	
4′			69.63(−1.60)	
5′			67.92(+1.21)	
6′			17.48(−0.17)	

TABLE 3. (Continued).

Carbon	Compound			
	5	7	8	9
X-1				101.67 (−0.36) [161.5]
2				71.16 (−0.40)
3				72.03 (−0.30)
4				69.41 (−0.16)
5				62.18[c] (−0.17)
CH_3CO	20.43 (3C)	20.44 (3C)	20.51 (3C)	20.54 (6C)
	20.58 (3C)	20.57 (5C)	20.58 (4C)	20.78 (3C)
	20.67	20.80 (3C)	20.77	21.01
	20.77	21.05 (2C)	20.88	
	21.10		21.10 (2C)	
CH_3CO	169.47	169.39	169.41	169.39
	169.67	169.58	169.78	169.52
	170.12	169.68 (2C)	169.92	169.67
	170.28 (4C)	170.17	170.12 (2C)	169.98
	170.49	170.29 (2C)	170.26 (3C)	170.12
	170.57	170.37 (2C)	170.59 (2C)	170.26 (3C)
		170.48	170.65	170.51
		170.55		170.74
		170.78		

[a]Values in parentheses denote downfield (+) or upfield (−) shifts as compared to methyl tri- or tetra-acetylglycoside.
[b]J ^{13}C-^{1}H (Hz).
[c,d]Assignments with the same superscript for each compound may be interchanged.
[e]Values in italics denote glycoside linkage carbon atoms.

sugar moiety of peracetate **5** were assigned by correlation with the fully assigned proton signals in the ^{1}H-^{13}C heteronuclear shift-correlated (HETCOR) 2D nmr spectrum (Table 3). The C-2 and C-4 signals (δ 76.54 and 77.58 ppm) in one glucose unit appeared shifted downfield by 4.55 and 8.43 ppm, respectively, relative to methyl *O*-tetraacetylglucoside. This evidence indicated that the glycosidic linkages were at the glucose 2 and 4 positions.

Enzymatic hydrolysis of trisaccharide **3** with cellulase followed by acetylation gave hexaacetate **6**, where the sugar side chain consisted of one glucose and one rhamnose (deduced from the ^{1}H-nmr spectrum). The proton signal of the glucose 2-position in the hexaacetate **6** was found shifted upfield by 1.34 ppm, relative to methyl *O*-tet-

TABLE 4. Fabms for Balanitins 4 [**1**], 5 [**2**], 6 [**3**], and 7 [**4**].[a]

Compound			
1	**2**	**3**	**4**
1069 $[M + Na]^+$	1053 $[M + Na]^+$	907 $[M + Na]^+$	1039 $[M + Na]^+$
1047 $[M + H]^+$	1031 $[M + H]^+$	885 $[M + H]^+$	1017 $[M + H]^+$
923 $[M + Na - 146]^+$	907 $[M + Na - 146]^+$	761 $[M + Na - 146]^+$	885 $(M + H - 132]^+$
901 $[M + H - 146]^+$	885 $(M + H - 146]^+$	739 $[M + H - 146]^+$	
			871 $[M + H - 146]^+$
907 $[M + Na - 162]^+$	739 $(M + H - 292]^+$	745 $[M + Na - 162]^+$	
885 $[M + H - 162]^+$		723 $[M + H - 162]^+$	761 $[M + Na - 278]^+$
	723 $[M + H - 308]^+$		739 $[M + H - 278]^+$
723 $[M + H - 324]^+$			
	578 $[M + H - 452]^+$		745 $[M + Na - 294]^+$
577 $[M + H - 470]^+$			723 $[M + H - 294]^+$

[a]Assignments of peaks are shown in brackets. The mass units which are lost correspond to the following fragments: 132, xylose; 146, rhamnose; 162, glucose; 278, xylose + rhamnose; 292, two rhamnose; 294, xylose + glucose; 308, rhamnose + glucose; 324, two glucose; 452, two rhamnose + glucose; 470, two glucose + rhamnose.

raacetylglucoside (Table 5). In turn this indicated that rhamnose was linked to the 2 position in glucose derivative **6**. Furthermore, the proton signals at the glycosidic linkage position were shifted upfield by ca. 1.2~1.5 ppm for glycosides **7–9** (Table 2). Based on this evidence, the structure of peracetate **5** was elucidated, and balanitin 6 was assigned structure **3**.

TABLE 5. ^{1}H-nmr Chemical Shifts and Coupling Constants of Balanitin 6 Hydrolyzate Peracetate [**6**] and Balanitin 7 Hydrolyzate Peracetate [**10**] in Pyridine-d_5.

Proton	**6**		**10**	
	δ ppm	J ^{1}H-^{1}H	δ ppm	J ^{1}H-^{1}H
H-3	3.93 m		3.93 m	
H-6	5.43[a]		5.43[a]	
H-16	4.55 m		4.55 m	
H-18	0.87 s		0.89 s	
H-19	1.10 s		1.10 s	
H-21	1.17 d	6.9	1.16 d	6.9
H-27	1.09 d	6.8	0.71 d	5.5
G-1	5.06 (+0.23)[b] d	7.8	5.06 (+0.23) d	7.8
2	4.12 (−1.34) dd	7.8, 9.6	4.12 (−1.34) dd	7.8, 9.6
3	5.83 (+0.10) t	9.6	5.83 (+0.10) t	9.6
4	5.44 (−0.06) t	9.6	5.44 (−0.06) t	9.6
5	4.12 (+0.02) ddd	3, 4.8, 9.6	4.12 (+0.02) ddd	3, 4.8, 9.6
6	4.42 (+0.02) dd	3, 12	4.42 (+0.02) dd	3, 12
	4.65 (+0.08) dd	4.8, 12	4.65 (+0.08) dd	4.8, 12
R-1	5.45 d	1.8	5.45 d	1.8
2	5.60 dd	1.8, 3.3	5.60 dd	1.8, 3.3
3	5.80 dd	3.3, 9.9	5.80 dd	3.3, 9.9
4	5.68 t	9.9	5.68 t	9.9
5	4.88 m	6.3, 9.9	4.88 dq	6.3, 9.9
6	1.50 d	6.3	1.50 d	6.3
MeCO	2.02		2.02	
	2.03		2.03	
	2.05 (6H)		2.05 (6H)	
	2.17		2.17	
	2.21		2.21	

[a]Overlapping signals.

[b]Values in parentheses denote downfield (+) or upfield (−) shifts as compared to methyl tetraacetylglucoside.

By application of the preceding structural approach, balanitin 4 [**1**] was shown to be a glycoside of yamogenin by analysis of the ir and ^{1}H- and ^{13}C-nmr spectra (Table 1). On acetylation steroidal saponin **1** gave dodecaacetate **7**. The peracetate ^{1}H-nmr spectrum indicated the presence of three glucose and one rhamnose units (Table 2). The fabms fragmentation of balanitin 4 suggested that rhamnose and glucose (bonded to a second glucose) were linked to glucose attached to yamogenin (Table 4). In the ^{13}C-nmr spectrum of the peracetate **7**, the C-2 and C-4 signals (δ 76.48 and 77.24 ppm) from one glucose unit and the C-3 signal (δ 79.77 ppm) of another glucose unit were shifted downfield by 4.49, 8.09, and 6.19 ppm, respectively (Table 3). Enzymatic hydrolysis of saponin **1** with naringinase followed by acetylation afforded balanitin 6 peracetate [**5**] and allowed assignment of structure **1** to balanitin-4.

That balanitin 5 [**2**] corresponded to a glycoside of yamogenin was deduced from the ir, ^{1}H- and ^{13}C-nmr spectra (Table 1). On acetylation saponin **2** afforded unde-

caacetate **8**, and the ^{1}H-nmr spectrum indicated the presence of two glucose and two rhamnose units (Table 2). Fragments in the fabms of glycoside **2** suggested that a rhamnose segment and a glucose unit attached to another rhamnose unit were in turn linked to glucose bonded to yamogenin (Table 4). The ^{13}C-nmr spectrum of peracetate **8** provided more useful data. The C-2 and C-4 signals (δ 76.53 and 77.33 ppm) of one glucose and the C-3 signals (δ 81.80 ppm) of another glucose appeared shifted downfield by 4.54, 8.18 and 8.22 ppm, respectively (Table 3). Enzymatic hydrolysis of steroidal saponin **2** with naringinase followed by acetylation gave peracetate **5**. The sum of this evidence led to structure **2** for balanitin 5.

In its ir spectrum, balanitin 7 [**4**] exhibited important bands at 982, 920, and 900 cm^{-1}. The 900 adsorption band was stronger than the 920 cm^{-1} band, typical of a 25(*R*)-spirostan (9, 10). In addition, the ^{1}H- and ^{13}C-nmr spectral data suggested that saponin **4** was a glycoside of diosgenin (Table 1). Acetylation of glycoside **4** gave undecaacetate **9**. The ^{1}H-nmr spectrum of the peracetate indicated the presence of two glucose, one rhamnose, and one xylose unit (Table 2). Application of fabms to saponin **4** suggested that the rhamnose unit and a glucose segment attached to xylose were each linked to glucose which in turn was bonded to diosgenin (Table 4). Enzymatic takadiastase hydrolysis of **4** followed by acetylation provided hexaacetate **10**. General features of the ^{1}H-nmr spectrum of the peracetate were identical with those of peracetate **6** except that the proton signals of the aglycone corresponded to those of diosgenin (Table 5). Therefore, peracetate **10** was assumed to have a structure in which the yamogenin aglycone of saponin **6** was replaced by diosgenin. As expected in peracetate **9**, the C-2 and C-4 ^{13}C-nmr signals (δ 76.46 and 77.19) of one glucose unit and the C-3 signal (δ 80.48) of the second glucose unit were respectively shifted downfield by 4.47, 8.04, and 6.90 ppm (Table 3). The evidence summarized above allowed assignment of structure **4** to balanitin 7.

Balanitins 4–7 exhibited cytostatic activity against P-388 cultured cells as shown in Table 6. Further evaluation of these cell growth inhibitory plant constituents is in progress.

TABLE 6. Evaluation of Balanitins 4 [**1**], 5 [**2**], 6 [**3**], and 7 [**4**] Against the P-388 Lymphocytic Leukemia Cell Line.

Steroidal Saponin	P-388 (ED_{50} μg/ml)[a]
1	0.41
2	2.40
3	0.21
4	0.22
5-Fluorouracil (standard)	0.08

[a]DMSO was used as vehicle.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—General experimental procedures have been described previously (8). All mp's were determined employing a Yanagimoto micromelting point apparatus. Optical rotations were measured with a JASCO ORD UV-5 spectropolarimeter. Hplc separations were performed using a Waters ALC-200 instrument equipped with a differential refractometer (R 401) and Shim-pack PREP-ODS (25 cm × 20 mm i.d.). Each of the balanitins was colorless. Spectroscopic measurements were conducted with the following instruments: ir, Hitachi EPI-G2; nmr (^{1}H and ^{13}C), Varian XL-300; fabms, Zab-sc.

The three enzymes employed for cleavage of the glycoside bonds were obtained from commercial sources as noted at end of the acknowledgment section. These enzymes are known to hydrolyze the follow-

ing specific disaccharide linkages. The specificity of these hydrolytic cleavages has been confirmed by the following nmr studies, such as recorded here: for cellulase 1,6-glc-glc (16–19), 1,2-rha-glc (18), 1,3-glc-ara (19), 1,2-rha-ara (19), 1,6-xyl-glc (17), and 1,3-xyl-ara (19); for takadiastase 1,4-glc-rha (20), 1,4-glc-xyl (20,21), and 1,4-xyl-rha (22); for naringinase, 1,2-glc-xyl (21).

SEED EXTRACTIONS.—Dried, finely ground seeds (36 kg) of *B. aegyptiaca* (a voucher specimen of which is maintained by the ASU-CRI) were extracted with CH_2Cl_2-MeOH (1:1) (150 liters) at ambient temperature. The extract was separated into CH_2Cl_2 and aqueous phases on addition (25% by volume) of H_2O. The aqueous fraction was evaporated in vacuo and freeze-dried to give the PS active (ED_{50} 6.0 μg/ml) extract (1251.3 g).

SOLVENT PARTITION SEQUENCE.—The PS cell line active aqueous extract (542.5 g) was successively partitioned between MeOH-H_2O (9:1) and hexane, MeOH-H_2O (4:1) and CCl_4, and MeOH-H_2O (1:1) and CH_2Cl_2. Removal of solvents gave the hexane (350 mg), CCl_4 (30.2 g), CH_2Cl_2 (8 g), and H_2O (504.5 g) fractions. The remaining H_2O fraction was further partitioned between *n*-BuOH and H_2O (1:1) to afford 166.5 g and 337.1 g fractions, respectively, on evaporation.

ISOLATION OF BALANITINS 4 [**1**], 5 [**2**], 6 [**3**], AND 7 [**4**].—The *n*-BuOH extract (166.5 g, PS, ED_{50} 17 μg/ml) was subjected to steric exclusion chromatography on a Sephadex LH-20 column, using MeOH as eluent. The first fraction (96.5 g) was rechromatographed on a Sephadex LH-20 column, using CH_2Cl_2-MeOH (2:3) as eluent. The second fraction (79.4 g) from this step was chromatographed on a Si gel column previously washed (successively) with the lower layer of $CHCl_3$-MeOH-H_2O (65:35:10) and MeOH followed by gradient elution with $CHCl_3 \rightarrow$MeOH. Elution with $CHCl_3$-MeOH (9:1) gave two P-388 cytostatic fractions (0.743 g and 7.62 g). The first fraction (0.743 g) was subjected to hplc with MeOH-H_2O (9:1) to afford saponins **3** (76.7 mg) and **4** (61.7 mg). The second fraction (7.62 g) from the Si gel column was repeatedly chromatographed on a Si gel column treated as noted above. Elution with $CHCl_3$-MeOH (9:1) gave saponins **1** (0.196 g) and **2** (0.32 g).

BALANITIN 4 [**1**].—Needles: mp 271–272° (from MeOH); $[\alpha]^{20}D$ −61° (*c* = 0.90, pyridine); fabms *m/z* 1069 $[M(C_{51}H_{82}O_{22}) + Na]^+$; ir ν KBr max cm^{-1} 3380 (OH), 988, 920, 900 [intensity 920>900, (25S)-spiroketal]; and ^{1}H-nmr (C_5D_5N) ppm 0.83 (3H, s, H-18), 1.06 (3H, s, H-19), 1.09 (3H, d, *J* = 6.9 Hz, H-27), 1.16 (3H, d, *J* = 6.9 Hz, H-21), 1.78 (1H, d, *J* = 6.0 Hz, rha), 3.35, 5.34 (OH, CH-O-), 5.45 (1H, d, *J* = 4.0 Hz, H-6), 6.25 (1H, d, *J* = 2.0 Hz, R-1).

Acetylation of balanitin 4 [**1**] (22 mg) with Ac_2O/pyridine followed by chromatography on Si gel and elution with 0.5% MeOH in $CHCl_3$ afforded dodecaacetate **7** (15 mg) as colorless needles: mp 135–137° (from iPrOH), $[\alpha]^{20}D$ −59° (*c* = 1.1, $CHCl_3$); fabms *m/z* 1551 $[M(C_{74}H_{106}O_{34}) + H]^+$; and ir ν max ($CHCl_3$) cm^{-1} 1730 (OAc).

BALANITIN 5 [**2**].—Needles: mp 203–207° (from MeOH); $[\alpha]^{20}D$ −78° (*c* = 1.47, pyridine); fabms *m/z* 1030 $[M(C_{51}H_{82}O_{21}) + Na]^+$; ir ν max (KBr) cm^{-1} 3380 (OH), 990, 920, 900 [intensity 920>900, 25(S)-spiroketal]; ^{1}H-nmr (C_5D_5N) ppm 0.83 (3H, s, H-18), 1.04 (3H, s, H-19), 1.08 (3H, d, *J* = 7.2 Hz, H-27), 1.15 (3H, d, *J* = 6.9 Hz, H-21), 1.69 (3H, d, *J* = 6.3 Hz, R-6′), 1.75 (3H, d, *J* = 6.3 Hz, R-6″). Acetylation of saponin **2** (62 mg) with Ac_2O/pyridine followed by chromatography on Si gel and elution with $CHCl_3$ afforded undecaacetate **8** (51 mg) as colorless needles: mp 145–149° (from iPrOH); $[\alpha]^{20}D$ −51° (*c* = 1.28, $CHCl_3$); fabms *m/z* 1493 $[M(C_{72}H_{104}O_{33}) + H]^+$; ir ν max ($CHCl_3$) cm^{-1} 1740 (OAc).

BALANITIN 6 [**3**].—Needles: mp 278–280° (from MeOH); $[\alpha]^{20}D$ −89° (*c* = 0.67, pyridine); fabms *m/z* 907 $[M(C_{45}H_{72}O_{17}) + Na]^+$; ir ν max (KBr) cm^{-1} 3430, 3280 (OH), 988, 920, 900 [intensity 920>900, 25(S)-spiroketal]; ^{1}H-nmr (C_5D_5N) ppm 0.84 (3H, s, H-18), 1.06 (3H, s, H-19), 1.09 (3H, d, *J* = 6.9 Hz, H-27), 1.16 (3H, d, *J* = 6.9 Hz, H-21), 1.78 (3H, d, *J* = 6.3 Hz, R-6), 3.35–5.35 (OH, CH-O-), 5.23 (1H, d, *J* = 4.3 Hz, H-6), 6.28 (1H, d, *J* = 2.0 Hz, R-1).

When glycoside **3** (30 mg) was acetylated with Ac_2O/pyridine and the product chromatographed on Si gel (elution with $CHCl_3$) nonaacetate **5** (25 mg) was obtained as colorless needles, mp 132–134° (iPrOH); $[\alpha]^{20}D$ −54° (*c* = 1.47, $CHCl_3$); fabms *m/z* 1263 $[M(C_{63}H_{90}O_{26}) + H]^+$; ir ν max ($CHCl_3$) cm^{-1} 1730 (OAc).

BALANITIN 7 [**4**].—Needles, mp 273–280° (from MeOH), $[\alpha]^{20}D$ −83° (*c* = 0.83, pyridine); fabms *m/z* 1039 $[M(C_{50}H_{80}O_{21}) + Na]^+$; ir ν max KBr cm^{-1} 3380 (OH), 982, 920, 900 [intensity 920>900, 25(*R*)-spiroketal]; and ^{1}H-nmr (C_5D_5N) ppm 0.70 (3H, d, *J* = 5.5 Hz, H-27), 0.84 (3H, s, H-18), 1.06 (3H, s, H-19), 1.15 (3H, d, *J* = 7.2 Hz, H-21), 1.79 (1H, d, *J* = 6.3 Hz, R-6), 3.35–5.30 (OH, CH-O-), 5.29 (1H, d, *J* = 4.0 Hz, H-6), 6.26 (1H, d, *J* = 2.0 Hz, R-1).

The peracetate derivative of saponin **4** (32 mg) was prepared and purified as summarized above for balanitin 6 peracetate [**5**] to afford undecaacetate **9** (26 mg) as colorless needles: mp 137–140° (iPrOH);

$[\alpha]^{21}D -58°$ ($c = 1.37$, $CHCl_3$); fabms *m/z* [M($C_{51}H_{102}O_{33}$) + H]$^+$ 1479; ir ν max ($CHCl_3$) cm^{-1} 1740 (OAc).

ENZYMATIC HYDROLYSIS OF BALANITIN 4 [**1**] WITH NARINGINASE.—A suspension of glycoside **1** (28 mg) in EtOH (8 ml) and Na_2HPO_4/citric acid buffer (pH 4.0, 20 ml) was treated with naringinase (200 mg). The mixture was stirred at 37° for 8 days and extracted with *n*-BuOH. The *n*-BuOH solution was evaporated under reduced pressure, and the residue was acetylated with Ac_2O/pyridine. After purification by Si gel cc (elution with $CHCl_3$), balanitin 6 peracetate [**5**] was obtained (3.2 mg).

ENZYMATIC HYDROLYSIS OF BALANITIN 5 [**2**] WITH NARINGINASE.—A suspension of saponin **2** (21 mg) was treated with naringinase (200 mg) as described in the preceding experiment. Stirring at 37° was continued for 4 days. Following isolation and acetylation, 4 mg of balanitin 6 peracetate [**5**] was realized.

ENZYMATIC HYDROLYSIS OF BALANITIN 6 [**3**] WITH CELLULASE.—A suspension of saponin **3** (26 mg) in EtOH (8 ml) and Na_2HPO_4 citric acid buffer (pH 4.0, 20 ml) was treated with cellulase (200 mg). The mixture was stirred at 37° for 9 days and extracted with *n*-BuOH. The *n*-BuOH was evaporated in vacuo, and the residue was acetylated with Ac_2O/pyridine. Purification by Si gel cc (in $CHCl_3$) provided hexaacetate **6** (4.1 mg) as a colorless oil: fabms *m/z* [M($C_{51}H_{74}O_{18}$) + H]$^+$ 975; ir ν max ($CHCl_3$) cm^{-1} 1730 (OAc).

ENZYMATIC HYDROLYSIS OF BALANITIN 7 [**4**] WITH TAKADIASTASE.—The preceding experiment was repeated with saponin **4** (17 mg) and crude takadiastase (100 mg), except for stirring at 37° for 8 days. Hexaacetate **10** (5 mg) was isolated as a colorless oil: fabms *m/z* 975 [M($C_{51}H_{74}O_{18}$) + H]$^+$, and ir ν max ($CHCl_3$) cm^{-1} 1730 (OAc).

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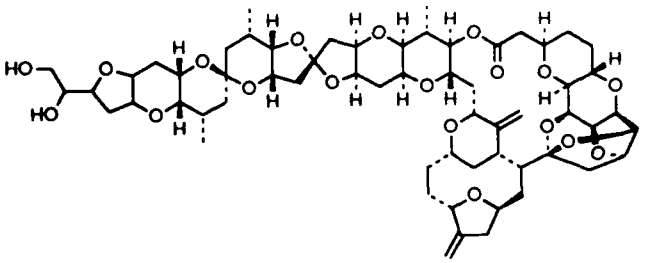
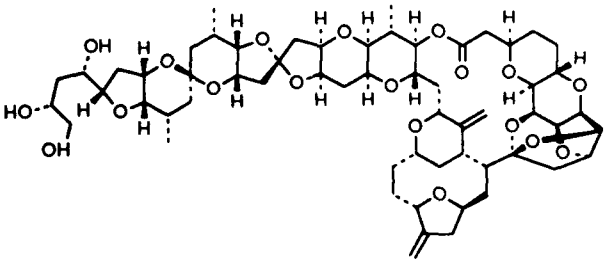
Isolation and Structure of the Cell Growth Inhibitory Constituents from the Western Pacific Marine Sponge *Axinella* sp.[1a]

While isolation of alkaloids from marine Porifera has been accelerating,[2] only a small number of antineoplastic[3] or peptide[3,4] constituents have been recovered from these invertebrates. Our isolation and structural determination of the P388 lymphocytic leukemia (PS system)[5] cell growth inhibitory cyclooctapeptide hymenistatin 1[4] from a Palau sponge in the genus *Hymeniacidon* represented the first such combination of source, structural type, and biological activity. We have also found an *Axinella* sp. (Demospongiae class) collected (in 1979) in Palau (at −40 m) to yield a methylene chloride–2-propanol extract that provided a 101% increase in life span (at 100 mg/kg) against the PS leukemia[5] with ED_{50} 2.5 μg/mL in the corresponding cell line.

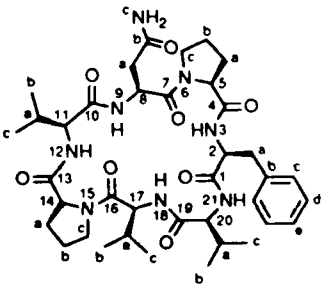
In 1985 the sponge was recollected (Palau) and preserved in 2-propanol. A 220-kg (wet weight) portion was extracted with methylene chloride–methanol. By means of PS guided bioassay and a series of detailed[3,4] solvent partition, gel permeation (and gel partition, Sephadex LH-20), partition (silica gel including reversed phase), and adsorption column chromatographic techniques, a series of structurally diverse antineoplastic constituents were detected in this very productive sponge. We now report that the most potent in vivo components were established[6] as the polyether macrolides, homohalichondrin B (1, 900 μg, 4.1×10^{-8}% yield, PS T/C 285 at 150 μg/kg) and halichondrin B (2, 400 μg, 1.8×10^{-8}% yield, PS T/C 238 at 25 μg/kg), heretofore found in trace amounts in one difficultly accessible Japanese sponge.[7,8] A new PS inhibitory (ED_{50} 0.21 μg/mL) peptide (3, 100 mg, 4.54×10^{-6}% yield) designated axinastatin 1 was also isolated, accompanied by axinohydantoin[3] (30 mg) and hymenialdisine[3] (0.53 mg).



1
(homohalichondrin B)



2
(halichondrin B)



3
(axinastatin 1)

Axinastatin 1 (3) crystallized from methylene chloride: mp 283–7 °C dec; $[\alpha]^{25}_D$ −161.6° (*c* 0.099, CH_3OH); TLC (R_f 0.18 in 95:5 CH_2Cl_2/MeOH); UV (CH_3OH) λ_{max} 208 nm (ε 18 000); IR (NaCl plate), ν_{max} 3320, 2960, 1640, 1520, 1465, 1430 cm^{-1}; high-resolution FAB MS 753.4293 $[M + H]^+$, theoretical mass for $[M + H]^+$ of $C_{38}H_{56}N_8O_8$ requires

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(6) By high-resolution FAB, NMR (2D, 400 MH_z) and comparison with an authentic sample of polyether 2 provided by Prof. D. Uemura.

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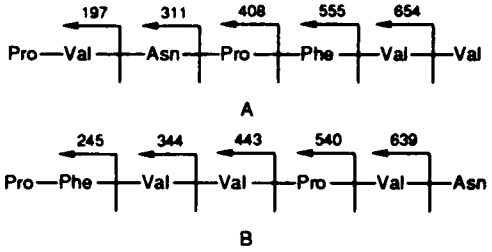
0022-2623/91/1834-3339$02.50/0

753.4299; amino acid analyses Asp, (or Asn), Phe, Pro, and Val in the ratio 1:1:2:3. The molecular formula for axinastatin 1 (3) was deduced from high-field (400 MHz) ^1H and ^{13}C NMR studies (see Table I of the supplementary material) in conjunction with the high-resolution FAB MS peak matching experiments just noted. Combined ^1H, ^1H COSY, ^1H, ^{13}C COSY, ^1H, ^1H relayed COSY,[9a] HMBC,[9b] and NOESY experiments confirmed the amino acid sequence and cyclic structure 3. The amino acid components and sequence of axinastatin 1 were confirmed as *cyclo*-(Asn-Pro-Phe-Val-Val-Pro-Val) by tandem (MS/MS) mass spectrometry.[10]

Protonation upon FAB results in ring opening of the cyclic peptide at an N-acyl bond to give a linear acylium ion.[10] The major fragmentation processes observed by tandem mass spectrometry involve losses of amino acid residues from the C terminus. Protonation is favored at proline, and with axinastatin 1 there are two possibilities. The FAB MS/MS spectrum of the [M + H] species contains two series (A and B) of ions resulting from protonation at the two proline units. All of the ions in both series



were observed. Additional supporting information for the sequence was obtained by MS/MS experiments on source-produced fragment ions to confirm the interrelationship of the fragment ions and by exact mass measurements on the fragment ions to verify elemental composition and correct assignment.

The absolute configuration of cycloheptapeptide 3 was ascertained by analyzing the acid hydrolysate *N*-pentafluoropropionyl–isopropyl ester[4] derivatives using chiral GC (Chirasil-Val III column). Each amino acid was found to have the L configuration. The disproportionatly high representation of L-Pro and L-Val in axinastatin 1 (3) and other strongly antineoplastic peptides[4] we have discovered in marine animals suggests that the presence of these amino acids may be an important structural requirement for controlling cell growth in peptide mediated systems.

The halichondrins proved to be remarkably potent against all of the 60 cell lines in the U.S. NCI's human tumor cell line in vitro screen,[11] yet with sufficient differences in relative sensitivity among the lines to yield a distinctive mean graph[12] profile. For halichondrin B and homohalichondrin B, the log molar GI_{50}'s for each line ranged from −8.10 to −9.70 and −8.05 to −10.08, respectively. The mean log molar GI_{50}'s were −8.95 and −8.99 for halichondrin B and homohalichondrin B, respectively. The characteristic mean graph "fingerprints" of the halichondrins were very similar; an analysis by the COMPARE pattern-recognition algorithm[13] showed their mean graph profiles to be most highly correlated to those produced by structurally unrelated, tubulin-binding standard agents[11] such as vincristine and taxol.

Discovery of the halichondrins in an *Axinella* sp., a sponge unrelated to their original source,[7] suggests that these exceptionally active[14] Porifera constituents may have a microorganism source. Either by exogenous and/or endogenous biosynthetic processes the marine porifera continue to be an especially fruitful source of potentially useful antineoplastic substances of novel structure.

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Supplementary Material Available: NMR spectra of axinastatin 1 and interpretation of tandem MS–MS spectra (28 pages). Ordering information is given on any current masthead page.

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Synthetic conversion of bryostatin 2 to bryostatin 1 and related bryopyrans[1]

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Bryostatin 2 (**1***a*) has been converted to bryostatin 1 (**1***e*) and bryostatin 12 (**1***i*) by a selective protection and deprotection involving the C-26 hydroxyl group. The new bryostatins **1***g*, **1***k*, and **1***m* were also prepared starting from bryostatin 2. The C-7 substituents of natural bryostatins 4 and 5 were revised from isovalerate → pivalate employing comparative ^{1}H and ^{13}C NMR studies of the semi-synthetic bryostatins **1***k* and **1***m* and the natural products.

Key words: bryostatin 2 → 1, selective conversion, bryostatins 4 and 5, pivalates.

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On a transformé la bryostatine 2 (**1***a*) en bryostatine 1 (**1***e*) et en bryostatine 12 (**1***i*) grâce à une protection et une déprotection sélective impliquant le groupement hydroxyle en C-26. On a aussi préparé les bryostatines **1***g*, **1***k* et **1***m* à partir de la bryostatine 2. En se basant sur des études comparatives de RMN du ^{1}H et du ^{13}C, effectuées sur les bryostatines semi-synthétiques **1***k* et **1***m* et sur les produits naturels, on propose une révision de la nature de substituants en position C-7 des bryostatines 4 et 5 d'isovalérate à pivalate.

Mots clés : bryostatine, conversion sélective, bryostatines 4 et 5, pivalates.

[Traduit par la rédaction]

The marine Bryozoan *Bugula neritina* has been found to contain a series of biologically and chemically exciting constituents now known as the bryostatins (2*a*–*g*). Other interesting biosynthetic products of the Phylum Bryozoa such as the β-lactam bearing chartellines have recently been isolated from *Chartella papyracea* (3). Bryostatin 1 (**1***e*) has been found to profoundly affect protein kinase C at picomolar concentrations (4*a*). Uniquely among protein kinase C modulators, whereas bryostatin 1 activates protein kinase C in vitro, it antagonizes phorbol ester responses in many biological systems (4*b*) and is deficient in tumor promoting activity (4*c*,*d*). Bryostatin 1 has powerful immunopotentiating activities (4*e*,*f*). The ability of bryostatin 1 to initiate cytotoxic T lymphocyte development (4*f*), and to induce production of interleukin-2 (4*f*,*g*) and growth of normal bone marrow cells (4*h*), combined with its strong *anti*tumor (4*i*) promoter and antineoplastic effects resulted in its selection for clinical development by the U.S. National Cancer Institute. To increase significantly the availability of bryostatin 1 (**1***e*) it became very important to convert bryostatin 2 (**1***a*), which was also obtained from *Bugula neritina* in nearly equal amounts, to bryostatin 1 in an efficient and selective way. Realization of this objective was accomplished as follows.

The bryostatin hydroxyl groups at C-3, C-9, and C-19 were earlier found (2*b*) to resist acetylation (under mild conditions), presumably due to intramolecular hydrogen bonding, while the C-7 and C-26 hydroxyl groups were readily acetylated (2*b*,*g*). Thus, synthetic conversion was undertaken by methods utilizing selective protection of the C-26 hydroxyl group. The steric environment of the two hydroxyl groups (viz. C-7 and C-26) suggested that a bulky silyl ether[3] would offer an attractive possibility.

Application of *tert*-butyldimethylsilyl chloride (6) was found very effective for selective protection of the bryostatin 2 C-26 hydroxyl group. Bryostatin 2 (**1***a*) was allowed to react (7) at room temperature with excess *tert*-butyldimethylsilyl (TBDMS) chloride in the presence of 4-(*N*,*N*-dimethyl)aminopyridine (and triethylamine in dimethylformamide) to produce 26-*tert*-butyldimethylsilyl ether **1***b* and bryostatin 2 7,26-di-*tert*-butyldimethylsilyl ether **1***c*. The disilyl ether was reconverted to bryostatin 2 employing 48% hydrofluoric acid – acetonitrile (1:20) (8). The yield of monosilyl ether **1***b* was 71% on the basis of total recovered bryostatin 2. Treatment of the C-26 silyl ether with acetic anhydride – pyridine (room temperature) gave acetate **1***d*. The C-26 hydroxyl was regenerated using 48% hydrofluoric acid – acetonitrile (1:20 at 0–5°C). The product was isolated in 80% overall yield (from **1***a*) by silica gel column chromatography and found to be identical with natural bryostatin 1 (**1***e*).

The high resolution SP-SIMS spectrum of bryostatin **1***e* displayed *m*/*z* 911 $(M + Li)^+$ and 927 $(M + Na)^+$ corresponding to the molecular formula $C_{47}H_{68}O_{17}$. The ^{1}H NMR spectrum revealed an acetate chemical shift at δ 2.04, the C-7 proton signal at δ 5.14 (dd, J = 12, 4.9 Hz), and the three-proton doublet of the C-27 methyl at δ 1.23 (J = 6.5 Hz). The significant downfield shift of the C-7 proton from δ 3.95 (**1***a*, **1***b*) to δ 5.14 (**1***e*) further confirmed acetylation at the C-7 hydroxyl group.

Selective protection of the C-26 hydroxyl group in bryostatin 2 allowed us to selectively introduce other groups at C-7.

[1]Part 192 of Antineoplastic Agents. For part 191 see ref. 1.

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[3]For review see Use of organosilicon reagents as protective groups in organic synthesis, ref. 5.

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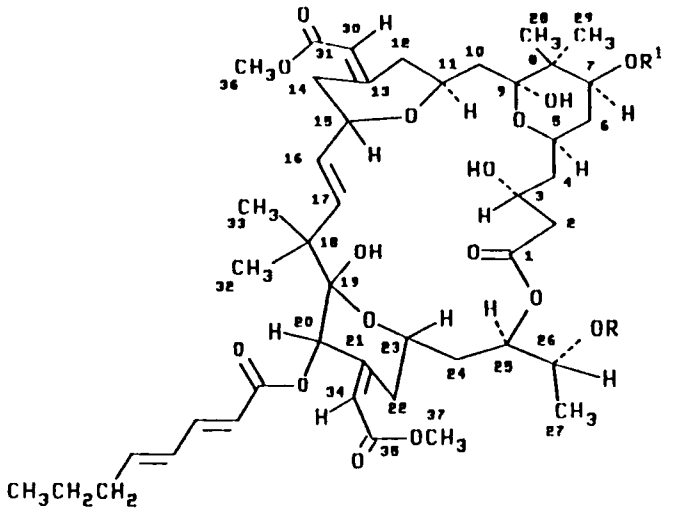


1, *a* R = R^1 = H, bryostatin 2
b R = TBDMS, R^1 = H
c R = R^1 = TBDMS
d R = TBDMS, R^1 = -$COCH_3$
e R = H, R^1 = -$COCH_3$, bryostatin 1
f R = TBDMS, R^1 = -$COCH_2CH_3$
g R = H, R^1 = -$COCH_2CH_3$
h R = TBDMS, R^1 = -$COCH_2CH_2CH_3$
i R = H, R^1 = -$COCH_2CH_2CH_3$, bryostatin 12
j R = TBDMS, R^1 = -$COCH_2CH(CH_3)_2$
k R = H, R^1 = -$COCH_2CH(CH_3)_2$
l R = TBDMS, R^1 = -$COC(CH_3)_3$
m R = H, R^1 = -$COC(CH_3)_3$

Treatment of 26-*tert*-butyldimethylsilyl ether **1***b* with butyric anhydride and pyridine, followed by deprotection, led to bryostatin 2 7-butyrate (**1***i*) identical to bryostatin 12 (**1***i*). Bryostatin 2 7-propionate (**1***g*) was obtained in an analogous manner.

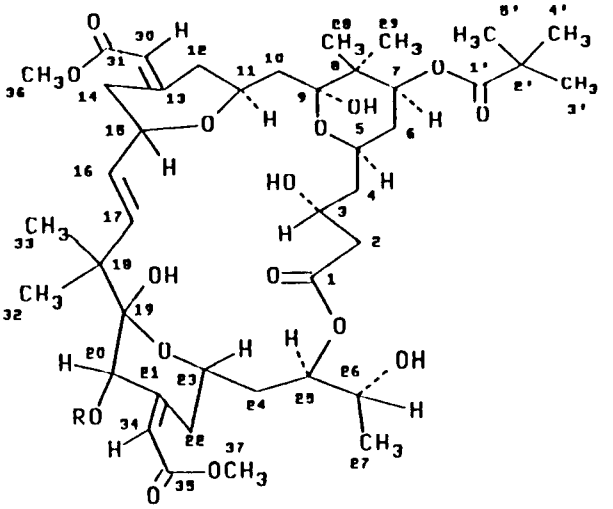
Conversion of bryostatin 2 to bryostatin 2 7-isovalerate (**1***k*) and bryostatin 2 7-pivalate (**1***m*) provided convincing support for the revision[4] of C-7 substituents in bryostatins 4 (**2***a*) (2*d*) and **5** (**2***b*) (2*e*). Esterification of bryostatin 2 26-OTBDMS (**1***b*) with isovaleric acid in the presence of dicyclohexylcarbodiimide and 4-pyrrolidinopyridine (10) in methylene chloride provided ester **1***j*. The TBDMS protecting group was removed to afford the new bryostatin **1***k*. Treatment of bryostatin 2 26-OTBDMS (**1***b*) with pivalic anhydride and 4-(*N*,*N*-dimethyl)aminopyridine (50–55°C) (for a review, see ref. 11) in methylene chloride provided bryostatin 2 26-OTBDMS-7-pivalate (**1***l*). Removal of the protecting group afforded bryostatin 2 7-pivalate (**1***m*) in 42% overall yield from **1***b*. The ^{1}H NMR spectrum of bryostatin **1***m* showed a strong singlet for the pivalate unit at δ 1.17 (Table 1) and a multiplet for the C-7 proton at δ 5.08 (dd, J = 11.8, 4.8 Hz), whereas bryostatin **1***k* showed a doublet at δ 0.93 (J = 6.3 Hz, Table 1) for the isovalerate unit.

The isovalerate → pivalate revision was further supported by the ^{13}C NMR spectra of natural bryostatins 4 and 5 (Table 2).

[4]In 1988, during another reisolation of bryostatins 4 and 5, recharacterization employing high-field NMR techniques suggested possible presence of a pivalate ester at C-7. The realization of this minor modification at C-7 was made possible by newer NMR techniques and these structural changes have already been entered in a contribution by Pettit *et al.* (9).

Comparison of the ^{1}H and ^{13}C NMR spectra of natural bryostatins 4 and 5 with those of the semi-synthetic bryostatins **1***m* and **1***k* confirmed the C-7 pivalate substitution. The strong singlet at δ 1.17 in the ^{1}H NMR spectrum of semi-synthetic bryostatin **1***m* (Table 1) was nearly identical to the singlet at δ 1.16 and δ 1.19 of natural bryostatins 4 and 5, respectively. Moreover, the bryostatin **1***m* gave rise to ^{13}C chemical shifts at δ 177.99 (C-1′), 39.01 (C-2′), and 27.14 (C-3′, -4′, and -5′, Table 2), in close agreement with the ^{13}C NMR shifts displayed by the C-7 substituents of natural bryostatins 4 and 5. The proton spectrum of bryostatin **1***k* showed a doublet due to two methyl groups at δ 0.93 (J = 6.3 Hz) (Table 1) and the ^{13}C NMR spectrum gave evidence of C-1′ at δ 172.81, C-2′ at δ 43.75, C-3′ at δ 24.93, and C-4′ and -5′ at δ 22.35 (Table 2), thereby substantiating that bryostatins 4 (**2***a*) and 5 (**2***b*) contain the unusual pivalate ester group at C-7 (2*d*,*e*, 9).

In initial biochemical characterization, the binding affinities of bryostatins **1***g*, **1***k*, and **1***m* for protein kinase C were determined by competition of [^{3}H]bryostatin 4 binding as described previously for bryostatins 1–10 (4*i*). The assay conditions involve reconstitution of protein kinase C in Triton X-100/phosphatidylserine. Although these conditions give



2, *a* R = -$COCH_2CH_2CH_3$, bryostatin 4
1″ 2″ 3″ 4″

b R = -$COCH_3$, bryostatin 5
1″ 2″

significantly lower affinity than found for the enzyme reconstituted under the more physiological conditions of phospholipid alone, they permit quantification under equilibrium conditions. The K_i values for bryostatins **1***g*, **1***k*, and **1***m* were 1.7 ± 0.5, 0.74 ± 0.23, and 0.73 ± 0.17 nM (mean ± range, n = 2 experiments) respectively. Similar values were thus observed for the isovalerate and pivalate esters, with slightly less activity for the propionate ester **1***g* and the acetate ester (bryostatin 1, K_i = 1.4 ± 0.2 nM, 4*i*).

The strategy for selective reaction at the C-7 hydroxyl group of bryostatin 2 with protection and deprotection of the more reactive C-26 hydroxyl now opens a useful pathway to a variety of new bryostatins (12). In time, that will allow further study of the structure/activity relationships among this fascinating group of marine animal antineoplastic constituents.

TABLE 1. Bryostatin ^{1}H NMR (400 MHz) chemical shift assignments in deuteriochloroform solution[a]

	Bryostatin 1*g*			Bryostatin 1*k*			Bryostatin 1*m*		
H	δ	Multiplicity	$J_{H,H}$ (Hz)	δ	Multiplicity	$J_{H,H}$ (Hz)	δ	Multiplicity	$J_{H,H}$ (Hz)
2	2.45	m		2.45	m		2.45	m	
3	4.16	m		4.17	m		4.17	m	
4	2.01, 1.56	m		2.06, 1.58	m		2.05, 1.58	m	
5	4.22	d	12	4.22	d	12	4.21	d	11.4
6	1.45, 1.75	m		1.44, 1.71	m		1.43, 1.71	m	
7	5.14	dd	11.8, 4.8	5.14	dd	11.8, 4.7	5.08	dd	11.8, 4.8
10	2.06, 2.15	m		2.07, 2.17	m		2.07, 2.14	m	
11	3.79	m		3.78	m		3.78	m	
12	2.06, 3.64	m		2.07, 3.63	m		2.06, 3.63	m	
14	1.86, 3.62	m		1.93, 3.63	m		1.89, 3.63	m	
15	4.04	dt	8.5, 2	4.03	m		4.04	m	
16	5.30	dd	15.8, 8.5	5.30	dd	15.7, 8.4	5.30	dd	15.7, 8.4
17	5.79	d	15.8	5.78	d	15.7	5.77	d	15.7
20	5.16	s		5.18	s		5.19	s	
22	2.08	m		2.09	m		2.09	m	
	1.66	d	15.1	1.67	d	15.2	1.64	d	15.1
23	4.01	m		4.01	m		4.00	m	
24	1.92, 2.06	m		1.91, 2.07	m		1.92, 2.07	m	
25	5.14	m		5.15	m		5.17	m	
26	3.79	m		3.80	m		3.80	m	
27	1.23	d	6.6	1.22	d	6.4	1.22	d	7.0
28[b]	0.93	s		0.92	s		0.93	s	
29[b]	0.99	s		0.99	s		0.99	s	
30	5.67	s		5.66	s		5.67	s	
32[c]	1.14	s		1.14	s		1.13	s	
33[c]	1.08	s		0.99	s		0.99	s	
34	6.00	d	1.5	5.99	d	1.5	5.99	d	1.5
36	3.69	s		3.69	s		3.68	s	
37	3.65	s		3.65	s		3.64	s	
2′	2.31	q	6.8	2.16	m		1.17	s	
3′	1.12	t	7.6	~1.9–2.0	m				
4′, 5′				0.93	d	6.3	1.17	s	
2″	5.80	d	15.4	5.80	d	15.2	5.80	d	15.2
3″	7.25	m		7.26	m		7.25	m	
4″	6.16	dd	8.5, 2.4	6.16	dd	8.5, 2.6	6.15	dd	8.2, 2.4
5″	6.16	dd	4.9, 1.5	6.16	dd	4.8, 1.5	6.16	dd	4.9, 1.5
6″	2.15	m		2.15	m		2.14	m	
7″	1.45	m		1.45	m		1.45	m	
8″	0.91	t	7.2	0.91	t	7.4	0.90	t	7.4

[a]Residual $CHCl_3$ as internal reference (7.25 ppm).
[b]Chemical shift values are interchangeable.
[c]Chemical shift values are interchangeable.

Experimental section

General procedures

Solvent solutions from reaction mixtures washed with water were dried over anhydrous sodium sulfate. All chromatographic solvents were redistilled. Silica gel (E. Merck, Darmstadt, 70–230 mesh) was employed for column chromatography and silica gel GHLF uniplates (Analtech, Inc., Newark, DE) were used for thin-layer chromatography (TLC). The TLC plates were viewed with UV light and developed with anisaldehyde – sulfuric acid spray reagent followed by heating. The NMR spectra were measured using a Bruker AM-400 instrument with deuteriochloroform employed as solvent. All high and low resolution fast atom bombardment (FAB) mass spectra (13) were recorded using a Kratos MS-50 mass spectrometer (Mid West Center for Mass Spectrometry, University of Nebraska, Lincoln, NE).

Bryostatin 2 26-tert-butyldimethylsilyl ether (1b). General procedure

The following procedure for silyation, acylation, and desilyation was repeated in analogous fashion for each bryostatin interconversion. A solution of bryostatin 2 (**1***a*, 50 mg), 4-(*N*,*N*-dimethyl)aminopyridine (15 mg), *tert*-butyldimethylsilyl chloride (40 mg), and triethylamine (20 μL) in dimethylformamide (2 mL) was stirred at room temperature (under argon) for 22 h. The reaction mixture was diluted with ice water, stirred for 10 min, and extracted with methylene chloride. The organic phase was washed with saturated aqueous sodium bicarbonate, followed by water, dried, and solvent evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (1:1 hexane – ethyl acetate) to afford silyl ether **1***b* (21.8 mg), bryostatin 2 7,26-di-*tert*-butyldimethylsilyl ether **1***c* (21.4 mg), and bryostatin 2 (5.5 mg). The silyl protecting groups in **1***c* were removed with 48% hydrofluoric acid – acetonitrile (1:20, 10 mL). The reaction mixture was stirred at 0–5°C (1.5 h), diluted with water, and extracted with methylene chloride. The chlorocarbon phase was washed with saturated aqueous sodium bicarbonate followed by water, and dried. The residue (from solvent removal at reduced pressure) was separated by silica gel column chromatography (1:1 hexane – ethyl acetate) to afford 17.2 mg of bryostatin 2. On the basis of total recovered bryostatin 2, the yield of monosilyl ether **1***b* was 71%. The 400 MHz ^{1}H NMR spectrum of silyl ether **1***b* displayed significant

TABLE 2. Bryostatin ^{13}C NMR chemical shift assignments in deuteriochloroform solution

C	Bryostatin 4 (**2***a*)	Bryostatin 5 (**2***b*)	Bryostatin **1***k*	Bryostatin **1***m*
1	172.21	172.31	172.15	172.11
2	42.14	42.14	42.35	42.37
3	65.45	65.50	65.79	65.79
4	39.94	39.89	39.98	39.86
5	68.46	68.46	68.46	68.46
6	33.19	33.17	33.35	33.16
7	72.68	72.60	72.43	72.23
8	41.20	41.19	40.93	41.17
9	101.72	101.75	101.79	101.76
10	44.13	44.11	44.09	44.08
11	64.70	64.69	64.68	64.68
12	31.21	31.17	31.27	31.28
13	157.25	157.09	156.42	156.41
14	36.47	36.40	36.32	36.31
15	78.88	78.92	79.12	79.12
16	129.67	129.67	129.39	129.38
17	138.96	138.91	139.26	139.27
18	44.73	44.75	44.89	44.89
19	98.83	98.71	98.99	98.99
20	74.25	74.39	74.03	74.03
21	151.84	151.68	151.96	151.97
22	41.86	41.86	41.97	41.94
23	71.44	71.43	71.46	71.45
24	35.81	35.79	35.84	35.83
25	73.56	73.61	73.65	73.61
26	70.01	70.02	70.13	70.10
27	19.60	19.66	19.79	19.78
28	19.76	19.81	19.79	19.78
29	20.99	21.00	21.07	21.01
30	113.96	114.05	114.45	114.46
31	166.70	166.72	166.68	166.68
32	16.91	16.92	16.88	16.89
33	24.57	24.58	24.57	24.57
34	119.58	119.67	119.56	119.57
35	166.97	166.96	167.00	167.01
36, 37	51.03	51.02, 51.10	51.04	51.05
1′	178.33	178.27	172.81	177.99
2′	39.01	39.01	43.75	39.01
3′	27.07	27.09	24.93	27.14
4′, 5′	27.07	27.09	22.35	27.14
1″	171.98	169.32	165.58	165.58
2″	36.47	21.43	118.60	118.59
3″	18.16		146.35	146.33
4″	13.57		128.36	128.37
5″			145.49	145.50
6″			35.04	35.05
7″			21.85	21.02
8″			13.67	13.68

chemical shifts at δ 0.07 (s, 3H), 0.11 (s, 3H), 0.90 (s, 9H), 1.08 (d, 3H, J = 5.6 Hz), 3.65 (s, 3H), 3.68 (s, 3H), 3.73 (m, 1H), and 3.95 (m, 1H).

*Conversion of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1**b) to bryostatin 1 (**1**e)*

A solution of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1***b*, 1.6 mg) in acetic anhydride (100 μL) and pyridine (150 μL) was stirred for 18 h (room temperature), diluted with methanol, and stirred for an additional 30 min. Solvent was removed (reduced pressure) and the residue was chromatographed on a column of silica gel (1:1 hexane – ethyl acetate) to afford 1.2 mg (72%) of acetate **1***d*. The product (**1***d*) was subjected to desilylation by treating with 48% hydrofluoric acid – acetonitrile (1:20, 100 μL). The reaction mixture was stirred at 0–5°C (1.5 h), diluted with water, and extracted with methylene chloride. The organic phase was washed with saturated aqueous sodium bicarbonate, water, and dried. After solvent removal (reduced pressure) the residue was purified by silica gel column chromatography (1:1 hexane – ethyl acetate) to afford bryostatin 1 (**1***e*, 0.8 g, 80%), identical with the natural product (by comparison with TLC, analytical HPLC, SP-SIMS (13), and ^1H NMR).

*Conversion of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1**b) to bryostatin 2 7-propionate (**1**g)*

Bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1***b*, 2 mg) was treated with propionic anhydride (100 μL) – pyridine (130 μL) and stirred for 18 h at room temperature. The product was purified as described directly above to afford **1***f* (2.0 mg, 95%). Desilylation and purification as described above afforded bryostatin **1***g* (1.5 mg, 88%): for ^1H NMR data see Table 1.

*Conversion of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1**b) to bryostatin 12 (**1**i)*

Bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1***b*, 2.7 mg) was esterified (20 h) with butyric anhydride (100 μL) in pyridine (120 μL) employing the same procedure as utilized above to provide ester **1***h* (1.9 mg, 73%). Desilylation (150 μL of the 1:20 48% hydrofluoric acid – acetonitrile) as described above gave bryostatin 12 (**1***i*, 0.8 mg, 47%), identical to the natural product (by comparison with TLC, SP-SIMS (13), and ^{1}H NMR spectra.

*Conversion of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1**b) to bryostatin 2 7-isovalerate (**1**k)*

A solution of isovaleric acid (5 μL), *N,N*-dicyclohexylcarbodiimide (10 mg), bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1***b*, 4.6 mg), and 4-pyrrolidinopyridine (1.3 mg) in methylene chloride (150 μL) was stirred at room temperature for 5 h (under argon). The *N,N*-dicyclohexylurea was removed by filtration, and the filtrate was concentrated (reduced pressure). The residue was separated by column chromatography on silica gel (1:1 hexane – ethyl acetate) to afford isovalerate **1***j* (4.5 mg, 90%). Removal of the silyl group as summarized above (cf., **1***e*, 150 μL of the 1:20 reagent) afforded bryostatin 2 7-isovalerate (**1***k*, 2.2 mg, 55%); FAB-MS (DTT/DTE as matrix), *m/z*: 969 $[M + Na]^+$ for $C_{50}H_{72}O_{16}Na$ (20% of base peak), and 911 $[M + H - 2H_2O]^+$ as base peak; for the ^{1}H and ^{13}C NMR data refer to Tables 1 and 2.

*Conversion of bryostatin 2 26-*tert*-butyldimethylsilyl ether (**1**b) to bryostatin 2 7-pivalate (**1**m)*

To a solution of bryostatin 2 26-*tert*-butyldimethylsilyl ether (6.9 mg) in methylene chloride (150 μL) were added 4-(*N,N*-dimethyl)amino-pyridine (45 mg) and pivalic anhydride (40 mg). The reaction mixture was stirred at 52–55°C for 4 h (under argon) followed by addition of methanol at room temperature. The residue obtained following solvent removal was separated by column chromatography on silica gel (1:1 hexane – ethyl acetate) to afford pivalate **1***l* (5.7 mg, 76%). Desilylation was performed as described earlier to afford bryostatin **1***m* (2.8 mg, 56%): FAB-MS (3-NBA Lil as matrix), *m/z*: 969 $[M + Na]^+$ for $C_{50}H_{72}O_{16}Na$ (10% of base peak), 953 $[M + Li]^+$ for $C_{50}H_{72}O_{16}Li$ as base peak; for the ^{1}H and ^{13}C NMR data consult Tables 1 and 2.

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